DYNAMIC INTERACTIONS OF DENDRITIC CELLS

-adhesive and migratory properties-

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Dynamic interactions of Dendritic Cells –adhesive and migratory properties-
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DYNAMIC INTERACTIONS OF DENDRITIC CELLS

-adhesive and migratory properties–

Een wetenschappelijke proeve op het gebied van de Medische Wetenschappen

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Voor mijn ouders

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Chapter 1

Introduction

Adapted and updated from Book on mechanisms of T cell migration, Landes Biosciences Publishers, in press

DENDRITIC CELLS AND T CELLS

The immune system continuously guards the body for invading pathogens, like bacteria and viruses. This complicated defense system comprises many different cells, which orchestrate to protect us against these microorganisms. The first line of defense, also called innate immunity, is regulated by macrophages, Natural Killer cells (NK cells), monocytes, and Dendritic Cells (DCs), which interact with pathogens in a non-specific manner. Although both NK cells and DCs are players in the innate immune response, their bi-directional cross-talk results in their involvement in the specific immune response (Moretta, 2002). This second line of defense, the specific recognition of antigens is controlled by cells of the adaptive immune system, which includes T and B cells. After their development in the thymus and bone marrow respectively, naïve T cells and B cells circulate via the blood through peripheral lymphoid organs, where they encounter antigens, which are presented by the sentinels of the immune system, the DCs (Butcher & Picker, 1996; Banchereau & Steinman, 1998). DCs and their precursors are continuously produced from hematopoietic stem cells within the bone marrow (Liu, 2001; Shortman & Liu, 2002). From *in vitro* studies it became clear that human hematopoietic stem cells can differentiate in two different DC precursors, one from myeloid origin and the other from lymphoid origin (Fig. 1). The myeloid precursor further differentiates into Langerhans cells (LCs), Interstitial DCs, and myeloid DCs (MDCs). The lymphoid precursor most likely differentiates in plasmacytoid DCs (PDCs)(Spits *et al.*, 2000). After antigen encounter all distinct DC subtypes mature. The functional plasticity among the different DC subsets, which depends on cytokine and antigen exposure, hampers the definition of distinct functions to the different DC lineages.

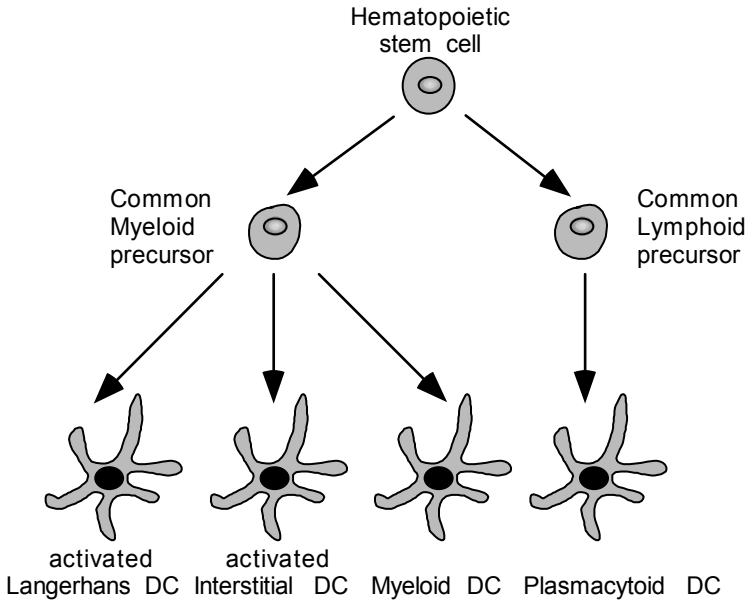


Figure 1. Proposed model for DC subsets.

Although many DC subsets can be generated *in vitro*, their relation with *in vivo* existing DCs still remains unclear. Studies using a transendothelial trafficking model point at monocytes,

especially the $CD16^+$ subclass, as precursors of DCs (Randolph *et al.*, 1998a; Randolph *et al.*, 2002). This is supported by an *in vivo* study, in which subcutaneously injected fluorescent microspheres are phagocytosed by monocytes, which subsequently migrate into the T cell areas of draining lymph nodes and express DC-specific markers (Randolph *et al.*, 1999). In the blood, three DC populations can be distinguished by specific blood DC markers, two of myeloid origin and one of lymphoid origin (Dzionek *et al.*, 2000; Grabbe *et al.*, 2000). The most commonly used DCs *in vitro* are derived from monocytes by culture in IL-4 and GM-CSF (Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994). Their maturation can be induced by several inflammatory mediators, like LPS or a combination of TNFα, IL-1β, PGE_2, and IL-6 (Jonuleit *et al.*, 1997).

DCs and their precursors enter the blood, transmigrate through the endothelium lining the blood vessel and populate the peripheral tissues. Here, DCs reside in an immature state and patrol for foreign antigens. Immature DCs are very efficient in antigen capture and can use either macropinocytosis, phagocytosis, or receptor-mediated endocytosis via Fcγ-receptors, toll-like receptors (TLRs), or lectins (Banchereau *et al.*, 2000; Figdor *et al.*, 2002; Gordon, 2002). Upon antigen uptake, DCs process the antigen into peptides, mature, and migrate through afferent lymph vessels into the T-cell areas of lymphoid organs to present the peptides and stimulate naïve T cells (Steinman, 1991; Banchereau & Steinman, 1998; De Vries *et al.*, 2003). Mature DCs are well equipped to present antigens; they express high levels of MHC class I and II molecules as well as the costimulatory molecules CD80 and CD86 and can be recognized by the expression of the maturation marker CD83.

Endogenous, cytosolic proteins processed to peptides by DCs are presented in MHC class I molecules to $CD8^+$, cytotoxic T cells, whereas exogenous derived peptides are presented in MHC class II molecules to $CD4^+$, helper T cells. $CD4^+$ T cells comprise Th1 cells and Th2 cells. Th1 cells are induced upon IL-12 production by DCs and can provide help to other T cells, like $CD8^+$ cells, whereas Th2 cells need IL-4 and can stimulate the production of antibodies by B cells. MDC and PDC stimulate in a cytokine-independent way the polarization of T cells to Th1 cells and Th2 cells, respectively (Rissoan *et al.*, 1999). Recently, it became clear that DCs are flexible in their ability to direct T cell polarization (Boonstra *et al.*, 2003). The plasticity of the DCs to direct Th1 or Th2 cell development is not directly linked to the DC subtype but is determined by the antigen dose, the maturation of the DCs, and the stimulation of DCs by pathogen-derived products. In general, high antigen doses stimulate Th1 cells, whereas low antigen doses induce Th2 cells.

Upon antigen recognition, T cells proliferate, differentiate into effector cells, and migrate from the lymph nodes via the blood to the side of inflammation, to provide help by regulating other cells or to attack pathogens (Fig. 2). Some of these T cells survive for a long period of time and become memory T cells. Based on their migratory capacity two subtypes of memory T cells can be distinguished; effector memory cells, which migrate to peripheral tissues, and central memory cells, which traffick to secondary lymphoid organs. After a second antigen challenge, central memory cells can stimulate DCs, provide help to B cells, and generate new effector cells (Sallusto *et al.*, 1999a).

To induce an immune response, DCs and T cells must migrate between peripheral tissues and the lymphoid organs. During this migration process, both DCs and T cells have multiple dynamic adhesive contacts with their environment. They interact with extracellular matrix (ECM) molecules in peripheral tissues and endothelium. The migration process is organ-specific and orchestrated by an intimate interplay between chemokines, chemokine

receptors, proteases and adhesion molecules, and establishes multiple attachments and detachments. The C-type lectin and integrin family of adhesion molecules are the main players involved in these contacts.

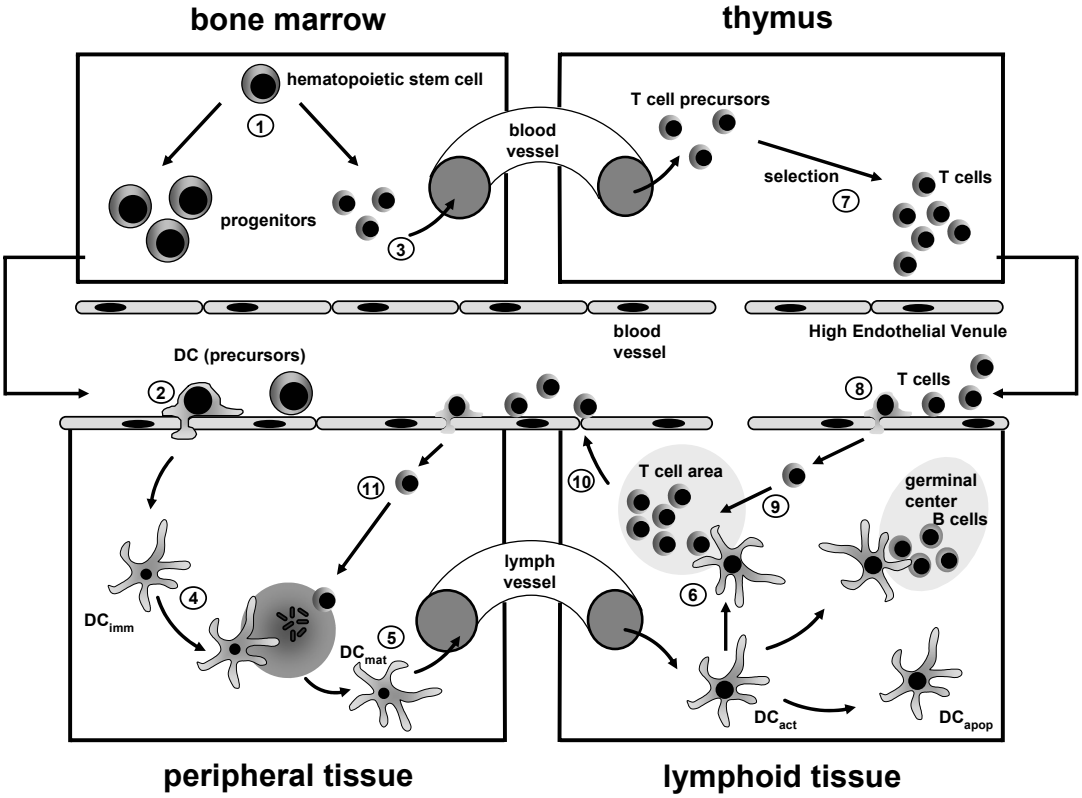


Figure 2. Migration of DCs and T cells. Hematopoietic stem cells differentiate in progenitor cells (1), which migrate via blood to peripheral tissues (2) or to the thymus (3) to differentiate into DCs and T cells. DCs and their precursors migrate from the blood into the peripheral tissue (2) and patrol as immature DCs for antigens. Upon antigen uptake (4), immature DCs process antigen, mature (5) and migrate via lymph vessels to the T cell area of the draining lymph node to stimulate naïve T cells (6). Progenitor cells that migrate from the bone marrow to the thymus undergo selection and become T cells (7). T cells migrate from thymus via High Endothelial Venules (HEV) into the lymph node (8) and subsequently also migrate into the T cell areas (9). After activation of T cells by DCs, T cells migrate via the blood vessel to the peripheral tissue (10) to exert their function (11).

C-TYPE LECTINS

In contrast to most cell adhesion molecules that establish protein-protein interactions, lectins bind sugars using carbohydrate recognition domains. One group of lectins are the C-type lectins, which bind their ligands in a calcium-dependent manner. The ligands are composed of a scaffold protein, or carrier molecule, which are modified by certain carbohydrates. The glycosylation of the ligand determines whether the ligand will interact with the receptor. Several subtypes of C-type lectins can be distinguished, like selectins and type I and type II lectins. Type I lectins have the N terminus outside the cell, whereas this terminus is pointing into the cytoplasm in type II lectins. Lectins are expressed on several cell types, including leukocytes and endothelial cells. They are involved in cell migration, in antigen uptake by

DCs, and in the interaction between DCs and T cells (Vestweber & Blanks, 1999; Geijtenbeek *et al.*, 2000c; Ariizumi *et al.*, 2000a; Geijtenbeek *et al.*, 2000a; Figdor *et al.*, 2002).

Table 1. Overview of C-type lectins, integrins, and their binding partners expressed on leukocytes and endothelium.

C-type Lectins 	**Ligands**
Selectins	
E-selectin (CD62E)	ESL-1, PSGL-1, sLe^x, sLe^a
P-selectin (CD62P)	PSGL-1, sLe^x, sLe^a, CD24
L-selectin (CD62L)	CD34, GlyCAM, MAdCAM, E-selectin
Type I receptors	
MMR	sLe^x
Type II receptors	
DC-SIGN	ICAM-2, ICAM-3, gp120, Ebola virus, SIV, CMV, hepatitis C virus, Leishmania amastigotes, Mycobacterium tuberculosis, Helicobacter pylori, Leishmania mexicana, Schistosoma mansoni, Candida Albicans, Le^x neoglycoconjugates
Integrins 	
β1 integrins	
α1β1 (CD49a/CD29, VLA-1)	Coll, LM
α2β1 (CD49b/CD29, VLA-2)	Coll, LM, E-cadherin
α3β1 (CD49c /CD29, VLA-3)	FN, LM, Coll, TSP-1
α4β1 (CD49d, VLA-4)	FN, VCAM-1, α4
α5β1 (CD49e /CD29, VLA-5)	FN
α6β1 (CD49f /CD29, VLA-6)	LM
α9β1	LM, VCAM-1, tenasin-C
β2 integrins	
αLβ2 (CD11a /CD18, LFA-1)	ICAM-1, -2, -3, -4, -5, JAM-1
αMβ2 (CD11b /CD18, Mac-1)	ICAM-1, -2, -4, uPAR, LPS, fibrinogen, C3bi, CD23, X, heparin and other soluble factors
αXβ2 (CD11c /CD18, p150,95)	fibrinogen, C3bi, CD23, X, LPS, and other soluble factors
αDβ2 (CD11d /CD18)	ICAM-3, VCAM-1
β3 integrins	
αvβ3	PECAM-1 (CD31), VN, fibrinogen
β7 integrins	
α4β7	FN, MAdCAM-1, VCAM-1, α4
αEβ7 (CD103)	E-cadherin

Lectins consist of 2 to 9 repeats of complement regulatory protein (○), an epidermal growth factor domain (▮), followed by a lectin domain (◀). Integrins are heterodimeric receptors that contain a non-covalently linked α and β domain. Abbreviations used: ESL-1, E-selectin ligand-1; PSGL-1, P-selectin glycoprotein ligand-1; sLe^x, sialyl lewis x; sLe^a, sialyl lewis a; GlyCAM, glycosylation-dependent cell adhesion molecule; MAdCAM, mucosal addressin cell adhesion molecule; MMR, macrophage mannose receptor; ICAM, intercellular adhesion molecule; DC-SIGN, DC-specific ICAM-3 grabbing non-integrin; SIV, simian immunodeficiency virus; CMV, cytomegalovirus; VLA, very late antigen; Coll, collagen; LM, laminin; FN, fibronectin; TSP-1, thrombospondin-1; VCAM-1, vascular cell adhesion molecule 1; LFA-1, leukocyte function-associated antigen-1; JAM-1, junctional adhesion molecule 1; uPAR, urokinase-type plasminogen activator receptor; LPS, lipopolysaccharide; C3bi, complement factor C3bi; X, factor X; PECAM-1, platelet-endothelial cell adhesion molecule-1; VN, vitronectin.

Selectins comprise L-, E-, and P-selectins (Vestweber & Blanks, 1999)(table 1). Whereas L-selectin is expressed on leukocytes, E-, and P-selectins are expressed by endothelial cells. Ligands for selectins include CD34, glycosylation-dependent cell adhesion molecule (GlyCAM-1), mucosal addressin cell adhesion molecule (MAdCAM-1), P-selectin glycoprotein ligand-1 (PSGL-1), E-selectin ligand-1 (ESL-1), and sialyl Lewis x (sLe^x). Selectins mediate rolling interactions between leukocytes and endothelium, which is the first step before adhesion and

subsequent migration of these leukocytes into the underlying tissue or lymph node (Springer, 1994). The relevance of the selectins in leukocyte homing is demonstrated in selectin-deficient mice, which have an increased susceptibility to bacterial infections (Tedder *et al.*, 1995; Frenette & Wagner, 1997).
Both type I and type II C-type lectins are expressed on DCs and LCs. The macrophage mannose receptor (MMR) and DEC-205 belong to the C-type I lectins and are involved in internalization of antigens (Sallusto *et al.*, 1995; Jiang *et al.*, 1995). MMR also mediates rolling interactions by binding to sLe^x structures on endothelial cells (Leteux *et al.*, 2000). Other receptors involved in antigen uptake are the C-type II lectins, Dectin 2 and DC-SIGN (DC-Specific ICAM-3 Grabbing Non-integrin) (Ariizumi *et al.*, 2000b; Geijtenbeek *et al.*, 2000a).
DC-SIGN mediates cellular interactions by binding to ICAM-2, ICAM-3, and Le^x neoglycoconjugates, which are non-sialylated (Geijtenbeek *et al.*, 2000a; Geijtenbeek *et al.*, 2000c; Appelmelk *et al.*, 2003)(table 1). Next to its function as an adhesion receptor, DC-SIGN binds to viral and non-viral pathogens, like HIV (Geijtenbeek *et al.*, 2000b), SIV (Pohlmann *et al.*, 2001), Ebola virus (Alvarez *et al.*, 2002; Simmons *et al.*, 2003), cytomegalovirus (Halary *et al.*, 2002), hepatitis C virus (Lozach *et al.*, 2003), dengue virus (Navarro-Sanchez *et al.*, 2003), Leishmania amastigotes (Colmenares *et al.*, 2002), Mycobacterium tuberculosis (Appelmelk *et al.*, 2003; Tailleux *et al.*, 2003), Helicobacter pylori, Leishmania mexicana, Schistosoma mansoni (Appelmelk *et al.*, 2003), and Candida Albicans (Cambi *et al.*, 2003). Moreover, DC-SIGN internalizes antigen for presentation to T cells and is an efficient DC-specific Ag receptor (Engering *et al.*, 2002b). *In vivo*, DC-SIGN is present on DCs in blood, in T cell areas of lymph nodes, tonsil, spleen, in dermis, and in mucosal tissues (Geijtenbeek *et al.*, 2000a; Soilleux *et al.*, 2002; Engering *et al.*, 2002a). Alveolar macrophages in the lung, specialized antigen-presenting cells, like Hofbauer cells in the placenta, and decidual macrophages also express DC-SIGN (Lee *et al.*, 2001; Geijtenbeek *et al.*, 2001; Soilleux *et al.*, 2001; Soilleux *et al.*, 2002; Kämmerer *et al.*, 2003). Furthermore, DC-SIGN expressing cells are found in atherosclerotic plaques (Soilleux *et al.*, 2002), in synovium of rheumatoid arthritis patients (van Lent *et al.*, 2003), and the colonic mucosa of patients with Crohn's disease (te Velde *et al.*, 2003). In mouse, five homologues of DC-SIGN are cloned, and only one is highly expressed on DCs (Park *et al.*, 2001). Murine DC-SIGN, designated as mSIGNR1, is specifically expressed by medullary and subcapsular macrophages in lymph nodes (Geijtenbeek *et al.*, 2002).

INTEGRINS

To date, the integrin family of glycoproteins comprises nineteen different integrin α-subunits and 8 different β-subunits that form at least 25 $\alpha\beta$ heterodimers (Hynes, 1992; Humphries, 2000). Based on shared β-subunits, eight subfamilies of integrins can be distinguished within the integrin family. The combination of an α-subunit with a β-subunit determines the binding specificity.
The $\beta1$ integrins are expressed on a wide variety of cell types, including hematopoietic cells, and mainly mediate interactions between cells and the ECM, such as collagen (Hemler, 1990), laminin (Mercurio, 1995; Taooka *et al.*, 1999), and fibronectin (Hemler, 1990; Petruzzelli *et al.*, 1999)(table 1). Some $\beta1$ integrins also mediate interactions between cells,

like $\alpha 2\beta 1$ and $\alpha 4\beta 1$. $\alpha 2\beta 1$ interacts with the adhesion molecule E-cadherin (Whittard *et al.*, 2002), expressed on epithelium, and $\alpha 4\beta 1$ also binds to $\alpha 4$ and VCAM-1 (vascular cell adhesion molecule 1), presented by endothelial cells (Altevogt *et al.*, 1995).
The $\beta 2$ and $\beta 7$ integrins are leukocyte-specific integrins, which mediate cell-cell interactions. The $\beta 2$ integrin family comprehends four molecules, $\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$, and $\alpha D\beta 2$ (table 1). The main ligands of these molecules, $\alpha X\beta 2$ excluded, are the InterCellular Adhesion Molecules (ICAM), which are members of the Ig superfamily. To date, five ICAMs have been identified. $\alpha L\beta 2$ binds to all five ICAMs, whereas $\alpha M\beta 2$ binds to ICAM-1, -2, and -4 (Marlin & Springer, 1987; Staunton *et al.*, 1989; Diamond *et al.*, 1991; Fawcett *et al.*, 1992; Tian *et al.*, 1997; Kotovuori *et al.*, 1999; Hermand *et al.*, 2000). $\alpha D\beta 2$ is a receptor for ICAM-3, but can also interact with VCAM-1 (Van der Vieren *et al.*, 1995; Van der Vieren *et al.*, 1999).
Integrins can exist in both an inactive as well as an active conformation on many cell types and need to be activated to exert their function (Hynes, 1992). Upon activation integrins change their conformation or cluster at the cell surface. This process is termed 'inside-out' signaling (Lub *et al.*, 1995). By this mechanism they regulate their binding to different ligands (van Kooyk & Figdor, 2000). Integrin activation can be induced by other cell surface receptors, like CD3 (van Kooyk *et al.*, 1989), phorbol esters that activate PKC (Rothlein & Springer, 1986; Danilov & Juliano, 1989; Shimizu *et al.*, 1990), G protein coupled signaling (e.g. via chemokines)(Constantin *et al.*, 2000; Grabovsky *et al.* 2000), or by particular activating antibodies (O'Toole *et al.*, 1990; van Kooyk *et al.*, 1991; Robinson *et al.*, 1992; van de Wiel-van Kemenade *et al.*, 1992; Arroyo *et al.*, 1992; Andrew *et al.*, 1993; Landis *et al.*, 1994). The intracellular signaling induced by these activation mechanisms regulate clustering of integrins and subsequent adhesion strengthening (Stewart *et al.*, 1998). Moreover, integrin-mediated adhesion requires the presence of divalent cations, like Ca^{2+}, Mg^{2+}, and Mn^{2+} (Gailit & Ruoslahti, 1988; Dransfield *et al.*, 1992). Activated $\alpha L\beta 2$ expresses a Ca^{2+}-dependent epitope, recognized by the NKI-L16 antibody (Keizer *et al.*, 1988), and a Mn^{2+}-dependent epitope, recognized by the M24 antibody (Dransfield *et al.*, 1992; Kamata *et al.*, 2002). The presence of the Ca^{2+}-dependent epitope on $\alpha L\beta 2$ corresponds with a clustered status of $\alpha L\beta 2$ and enables ligand binding (van Kooyk *et al.*, 1994; Binnerts & van Kooyk, 1999). Upon ligand binding or cross-linking of integrins, they induce 'outside-in' signals, such as increased levels of intracellular calcium and pH, phosphorylation of PLC, and costimulatory signals, which lead to biological responses, like cell spreading and migration (Wacholtz *et al.*, 1989; Figdor *et al.*, 1990; Van Seventer *et al.*, 1990; Hynes, 1992; Van Seventer *et al.*, 1992; Kanner *et al.*, 1993).
The functional importance of integrin families in the immune system has been illustrated by $\beta 2$-/- mice and by genetic defects in integrin subunits that abolish integrin-mediated adhesive functions *in vivo* (Scharffetter-Kochanek *et al.*, 1998). This is best illustrated in patients suffering from Leukocyte Adhesion Deficiency (LAD), which have lost expression of $\beta 2$ molecules due to deletions or mutations in this subunit (Anderson & Springer, 1987). LAD-patients die at a very young age of recurrent bacterial infections, because of impaired leukocyte adhesive functions.

CHEMOKINES

Chemokines are small secreted chemotactic cytokines, which signal through seven-fold membrane spanning G-protein-coupled receptors. Four families of chemokines can be distinguished based on cysteine motifs which connect the N-terminal loop to the more structured core of the molecule (C, CC, CXC, and CX_3C). There is a large diversity in signaling since one chemokine can interact with more receptors and one receptor can bind distinct chemokines. Several chemokine receptors are expressed on DCs and T cells (table 2). Chemokines are involved in leukocyte trafficking by regulating integrin-dependent adhesion of leukocytes to endothelium, are specialized in tissue-specific homing of T cells, and are involved in microenvironmental segregation of lymphocyte subsets in lymphoid organs. An overview of chemokine receptors expressed by monocytes, DCs, and T cells is presented in Table 2.

DCs and T cells have to pass the endothelium to enter peripheral tissues. Endothelial cells produce chemokines or transfer chemokines that are produced by other cells from the abluminal part to the lumen of the blood vessel to display them to leukocytes (Sallusto *et al.*, 2000). Some chemokines will be immobilized via heparan sulfate-binding domains to glycosaminoglycans expressed on endothelium (Gale & McColl, 1999). Upon encountering chemokines, integrins expressed on rolling leukocytes will be activated resulting in an integrin-mediated arrest of the leukocytes to the vessel wall. For instance, CCL19 (MIP-3β/ELC), CCL20 (MIP-3α/LARC), CCL21 (6C-kine/SLC), and CXCL12 (SDF-1α/β) rapidly enhance the adhesion of T cells to endothelium under flow conditions (Campbell *et al.*, 1998; Grabovsky *et al.*, 2000; Constantin *et al.*, 2000). Moreover, apical endothelial chemokines in combination with shear stress promote transendothelial migration of lymphocytes (Cinamon *et al.*, 2001).

Table 2. Overview of chemokine receptors expressed by monocytes, DCs, and T cells.

chemokine receptor	**chemokine**	**Expression of chemokine receptor**
CCR1	CCL3, CCL5, CCL7, CCL8, CCL13-16, CCL23	monocyte, immature DC, PDC, Th1, Tem
CCR2	CCL2, CCL7, CCL8, CCL13	monocyte, immature DC, MDC, PDC, Th1, Th2
CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26	MDC, PDC, Th2, Tem
CCR4	CCL17, CCL22	mature DC, MDC, PDC, Th2, semi-naïve T, Tcm, Tem
CCR5	CCL3-5, CCL8, CCL14	monocyte, immature DC, MDC, PDC, Th1, Tem
CCR6	CCL20	immature DC, memory/activated T, Tcm, Tem
CCR7	CCL19, CCL21	mature DC, MDC, PDC, mature MDC, mature PDC, naïve T, semi-naïve T, Tcm
CXCR1	CXCL8	monocyte, immature DC, MDC, PDC
CXCR2	CXCL1-3, CXCL5-8	MDC, PDC
CXCR3	CXCL9-11	MDC, PDC, Tem, Tcm
CXCR4	CXCL12	monocyte, mature DC, MDC, PDC, naïve T
CXCR5	CXCL13	Th1, Th2
unknown	CCL18	naïve T

Abbreviations used: PDC, plasmacytoid DC; MDC, myeloid DC; Th1, T helper 1 cells; Th2, T helper 2 cells; Tcm, central memory T cells; Tem, effector memory T cells.

Currently, no data are available about chemokine-induced arrest of DCs. However, the arrest of monocytes, which are DC-precursors, is mediated via CXCR1 and CCR2 (Gerszten *et al.*, 1999). The recruitment of immature DCs into inflamed tissue is mediated by CXCR1, CCR1, CCR2, and CCR5. During DC maturation these receptors are downmodulated, whereas CXCR4, CCR4, and especially CCR7 are upregulated. Hence, mature DCs become responsive to CCL19 and CCL21, which are produced by lymphatic endothelium and interdigitating DCs, respectively (Sallusto *et al.*, 2000). This maturation-induced expression of CCR7 is also observed for MDCs and PDCs (Penna *et al.*, 2001; Penna *et al.*, 2002; de la Rosa *et al.*, 2003). The pivotal role for CCR7 in DC migration to lymph nodes is demonstrated in CCR7 knock out mice, which show a defect in DC migration from the skin to the lymph nodes (Forster *et al.*, 1999).
Homing of naïve T cells to peripheral lymph nodes is also mediated by CCL19 and CCL21, which bind to CCR7 (Campbell *et al.*, 1998). Upon stimulation of T cells with antigen, CCR7 is downregulated and both CXCR5, and CCR4 are upregulated to make T cells more sensitive to chemokines produced in the B cell areas. Central memory T cells express CCR4, CCR6, and CXCR3, whereas effector memory T cells in addition to these receptors also express CCR1, CCR3, and CCR5 (Sallusto & Lanzavecchia, 2000).
DCs produce chemokines, some of which are induced upon maturation (Sallusto *et al.*, 1999b; Vissers *et al.*, 2001). The regulated expression of chemokines by DCs indicates that upon stimulation DCs first attrack immature DCs and other inflammatory cells, whereas at later stages they produce CCL18 (DC-CK1, PARC) and CXCL12 to recruit T cells into lymphoid organs to facilitate T cell activation (Adema *et al.*, 1997). The expression of chemokines by blood DCs as well as Langerhans cells resembles the expression by monocyte-derived DCs (Vissers *et al.*, 2001).

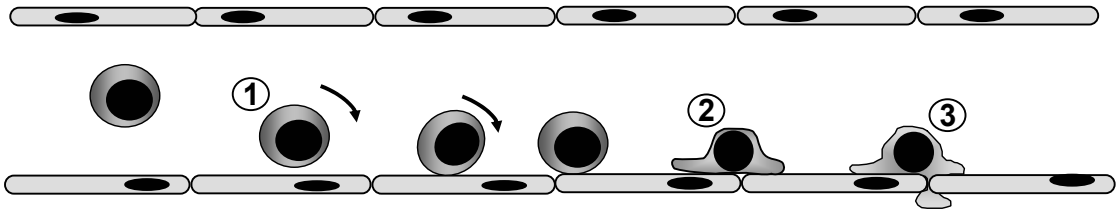
MULTI-STEP MIGRATION MODEL

To extravasate into (inflamed) tissues, (precursor) DCs and T cells first tether to endothelium, roll along the endothelial cell lining, adhere, and subsequently transmigrate through the endothelium into the underlying tissue (Fig. 3)(Butcher, 1991; Springer, 1994). This multi-step process requires sequential engagement of adhesion and signaling receptors. Though adhesion molecules have distinct expression patterns on different type of endothelial cells, they all mediate transendothelial migration of T cells and DCs, but use different ligand and receptor combinations. Whereas lectins are mainly involved in rolling interactions, integrins play a role in each event in this multi-step cascade (Fig. 3).

Rolling

The initial rolling step is mediated by selectins (E-, P-, and L-selectin) and their ligands, $\alpha 4$ integrins, and CD44 (Alon *et al.*, 1995; Berlin *et al.*, 1995; DeGrendele *et al.*, 1996; Chen *et al.*, 1999; Robert *et al.*, 1999). $\alpha L\beta 2$ can also mediate rolling when transfected in erythroleukemic K562 cells (Sigal *et al.*, 2000; Shimaoka *et al.*, 2002). However $\alpha L\beta 2$-mediated rolling is cell type-dependent, since wild type $\alpha L\beta 2$ in subsets of peripheral blood lymphocytes and Jurkat cells is unable to initiate rolling on ICAM-1 (Sigal *et al.*, 2000). Most likely, this difference in rolling capacity of $\alpha L\beta 2$ depends on the restriction in $\alpha L\beta 2$-mediated adhesion strengthening, which is cell-type dependent (Sigal *et al.*, 2000).

In vivo rolling depends on selectins and selectin-mediated rolling is more efficient than integrin-dependent rolling (Gaboury & Kubes, 1994; Lawrence *et al.*, 1995). The extravasation of DCs depends on fucosylated ligands of endothelial selectins, however, is not mediated by PSGL-1 (Robert *et al.*, 1999; Pendl *et al.*, 2002). DC-SIGN is also implicated in rolling interactions as was shown in an *in vitro* study in DC-SIGN-transfected erythroleukemic K562 cells (Geijtenbeek *et al.*, 2000c). This may imply that DCs use this molecule to roll on endothelium. During the tethering step, T cells and DCs will be exposed to stimuli causing activation-dependent firm adhesion.



		Leukocyte	blood vessel
1	**tethering/rolling**	L-selectin	Sialyated sugars, CD34, MAdCAM-1, Glycam-1, (E-selectin)
		sLe^x, sLe^a, PSGL-1	E-selectin, P-selectin
		$\alpha4\beta1$	VCAM-1
		$\alpha4\beta7$	MAdCAM-1, VCAM-1
		$\alpha L\beta2$	ICAM-1
		DC-SIGN	ICAM-2
		CD44	hyaluronan
2	**arrest/adhesion**	$\alpha L\beta2$	ICAM-1, ICAM-2
		$\alpha M\beta2$	ICAM-1, ICAM-2
		$\alpha4\beta1$	VCAM-1
		$\alpha4\beta7$	MAdCAM-1, VCAM-1
		$\alpha v\beta3$	CD31
		CD31	CD31
		?	VAP-1
3	**transmigration**	$\alpha L\beta2$	ICAM-1, ICAM-2, JAM-1
		JAM-2?	JAM-2
		$\alpha M\beta2$	ICAM-1, ICAM-2
		$\alpha4\beta1$	VCAM-1
		CD31	CD31
		CD99	CD99

Figure 3. Multi-step migration model. To extravasate into tissue, DCs and T cells tether and roll on endothelium (1), arrest and adhere (2), and finally transmigrate through the endothelial layer (3). Multiple adhesion receptors are involved in these different steps, among them the integrins and lectins. Molecules implicated in each step are depicted below the graph.

Arrest and adhesion

The transition from rolling to adhesion is characterized by the activation of integrins, in particular $\alpha L\beta2$ and $\alpha4\beta1$. Both, L-selectin and $\alpha4\beta7$ are required for engagement of $\alpha L\beta2$ with its ligand (Bargatze *et al.*, 1995). In contrast, L-selectin-mediated rolling is not mandatory for the adhesion of $\alpha4$ integrins (Berlin *et al.*, 1995). The importance of integrin activation is clearly demonstrated in experiments in which they locked the ligand binding domain (I domain) of $\alpha L\beta2$ in a closed or an open conformation, resulting in low or high

affinity for its ligand, respectively. Whereas wild type I-domain and wild type αLβ2 mediate rolling, the locked open form is involved in firm adhesion (Salas *et al.*, 2002). In other studies with non-stimulated Jurkat cells, α4β1 also supported rolling and spontaneous arrest on VCAM-1, whereas a reduced α4β1 affinity failed to mediate spontaneous arrest after tethering to or during rolling on VCAM-1 (Chen *et al.*, 1999). Thus, high affinity integrin receptors are pivotal in inducing arrest of rolling cells under flow.
Integrin activation can be accomplished by signaling via chemokines presented on endothelium or via ligand signaling and takes place in fractions of seconds (Grabovsky *et al.*, 2002). Immobilized chemokines can induce integrin-mediated capture of lymphocytes and arrest, but can also interfere with a subsecond stabilization of selectin tethers which are necessary for persistent rolling (Campbell *et al.*, 1998; Grabovsky *et al.*, 2000; Constantin *et al.*, 2000; Grabovsky *et al.*, 2002). Cross talk between integrins also induces activation of one of these integrins. For example, the activation status of α4β1 and subsequent ligation to VCAM-1 regulates the α4β1- and αLβ2-mediated adhesion and migration (Chan *et al.*, 2000; May *et al.*, 2000; Rose *et al.*, 2001). On the contrary, adhesion of human T cells to ICAM-1 via αLβ2 decreases the adhesion mediated via α4β1 to VCAM-1 and fibronectin, however, has less effect on α5β1-mediated binding (Porter & Hogg, 1997). This latter cross talk might be important for the transition of adherent to more migratory cells. Another example that adhesion molecules can induce integrin activation is the adhesion initiated by CD44 binding inducing α4β1-mediated firm adhesion and subsequent transmigration of activated T cells (Siegelman *et al.*, 2000).
Integrins involved in adhesion of leukocytes to endothelium are α4β1, α4β7 (Bargatze *et al.*, 1995), αMβ2 (Smith *et al.*, 1989), and αLβ2 (Bargatze *et al.*, 1995). The binding of T cells to resting endothelium is mainly mediated by αLβ2/ICAM-1 interactions. In contrast, adhesion to activated endothelium is partially accomplished via α4β1/VCAM-1 interactions (Oppenheimer-Marks *et al.*, 1991). α4β1-mediated adhesion to endothelium only occurs when αLβ2 is not present or inactive (Wysocki & Issekutz, 1992; van Kooyk *et al.*, 1993). The adhesion of DCs, monocyte-derived as well as blood DCs, to resting endothelium is mediated by DC-SIGN, β2 integrins, αLβ2 and αMβ2, whereas DC-SIGN, β2 integrins, and α4β1 are involved in the adhesion of DCs to activated endothelium (Brown *et al.*, 1997; D'Amico *et al.*, 1998; Bianchi *et al.*, 2000; Geijtenbeek *et al.*, 2000c).

Diapedesis

To extravasate into the underlying tissue, leukocytes have to transit from the firmly adherent state into a more migratory phenotype. As discussed above, this can be established by cross talk of αLβ2 to α4β1, resulting in a diminished adhesion to VCAM-1 (Porter & Hogg, 1997). The binding of αLβ2 and α4β1 to their endothelial and ECM counterparts can induce migration (Hauzenberger *et al.*, 1997). Moreover, subsequent internalization of VCAM-1 by the endothelium would facilitate de-adhesion of lymphocytes (Ricard *et al.*, 1998).
The final diapedesis requires αLβ2 binding to ICAM-1, ICAM-2, and JAM-1 (Butcher, 1991; Springer, 1994; Ostermann *et al.*, 2002), α4β1 binding to VCAM-1, homo- and heterotypic CD31 aggregation, possibly homo- or heterotypic JAM-2 interactions (Johnson-Leger *et al.*, 2002; JAM-2 has also been named JAM-3 by other groups) and homotypic CD99 interactions (Schenkel *et al.*, 2002). αLβ2/ICAM interactions are prominent over α4β1/VCAM-1 interactions (Roth *et al.*, 1995). The major role of αLβ2 in this process is illustrated in LAD

clones, which have lost expression of β2 molecules and migrate significantly less through an endothelial monolayer than control clones (Kavanaugh *et al.*, 1991). In addition, monocyte-derived DCs from LAD-1 patients showed an abnormal morphology and an impaired transendothelial migration, indicating that β2 integrins are involved in migration of DCs (Fiorini *et al.*, 2002). In transmigration of DCs through a microvascular endothelial monolayer DC-SIGN also plays a role (Geijtenbeek *et al.*, 2000c).
The sequence of steps in this final part of the transmigration process is not clear. αLβ2/JAM-1 interactions might act in concert with, prior to, or after CD31/CD31 ligation. However, CD99 is involved at the end of transmigration, just before this process is completed (Aurrand-Lions *et al.*, 2002). The diapedesis of DCs is, in contrast to the adhesion process, not only dependent on αLβ2, but also on CD31 ligation (D'Amico *et al.*, 1998). The reverse transmigration of DCs involves other proteins, like p-glycoprotein and tissue factor (Randolph *et al.*, 2002) and is mediated via the lipid transporter multidrug resistance protein 1 (MDR-1) and the LTC4 transporter multidrug resistance associated protein 1 (MRP1) (Randolph *et al.*, 1998b; Robbiani *et al.*, 2000). LTC4, a cysteinyl leukotriene (cysLT) is a key mediator of DC chemotaxis and lymphatic migration by triggering CCL19-dependent migration of DCs to lymph nodes.

Infiltration into tissue
After passing the endothelial monolayer and underlying basement membrane, DCs and T cells enter the tissue and interact mainly via β1 integrin receptors with the ECM. Transmigrated T cells possess lower expression levels of α4β1 and αLβ2, resulting in a decreased adhesion to VCAM-1 and ICAM-1, respectively. In addition, the adhesion of transmigrated T cells to ECM molecules, like fibronectin, collagen type I and IV was increased (Romanic *et al.*, 1997). The expression of α4β1 is lost when T cells migrate more deeply into the tissue, as was shown on brain sections of experimental autoimmune encephalomyelitis mice (Romanic *et al.*, 1997).

T CELL AND DC INTERACTIONS WITH ECM

During inflammation, cells produce inflammatory mediators, like cytokines and chemokines, which bind to the ECM and create a haptotactic gradient of chemoattractant (Schor *et al.*, 2000). In this way, the immune response is restricted to inflammatory regions. T cells exhibit a haptotactic (a directed response of cells in a gradient of adhesion) and chemotactic migration to laminin, fibronectin and collagen type IV (Hauzenberger *et al.*, 1995). However, two-photon imaging studies question the role of chemokine gradients within T cells areas, because T cells appear to migrate independent paths and do migrate in a random manner (Miller *et al.*, 2002).
Both T cells and DCs bind to the ECM via β1 integrins. Several integrins can bind to the same ECM structure (table 1). For instance, both α4β1 and α5β1 are receptors for fibronectin. Dependent on cell type and the process (adhesion or migration) both α4β1 and α5β1 or one of these integrins are involved in the interaction (Hauzenberger *et al.*, 1994; D'Amico *et al.*, 1998; Bianchi *et al.*, 2000; De Vries *et al.*, 2003). The induction of T cell migration to ECM molecules requires signaling via either α4β1 or αLβ2 (Hauzenberger *et al.*, 1995).

In vivo, ECM molecules are arranged in matrices. To mimic the *in vivo* situation, three-dimensional matrix models have been developed (Friedl *et al.*, 1993). The matrix forces the cells to adapt their morphology or degrade the ECM components by proteolytic enzymes (Friedl & Brocker, 2000). Indeed, the migration of T cells and DCs in collagen type I matrices is determined by the adaptation of the cell shape of the cells, and is β1 integrin-independent (Friedl *et al.*, 1998; Friedl & Brocker, 2000). Nevertheless, the migration of $CD4^+$ T cells was diminished by increased adhesion induced by β1 integrin-activating antibodies (Friedl *et al.*, 1998). Migration via non-specific low affinity interactions of T cells with either charged or uncharged residues of the matrix can not be excluded (Friedl & Brocker, 2000). T cells and DCs form short-lived transient interactions with collagen without remodeling the matrix (Friedl *et al.*, 1998). The matrix structure might therefore play a redundant role in T cell and DC migration (Friedl & Brocker, 2000). Although *in vitro* studies in collagen matrix models resemble DC-T cell interactions as determined by two photon imaging of T cells in lymph nodes *in vivo*, mean migration velocities of these T cells are less when investigated in collagen matrix models (Friedl *et al.*, 1994; Miller *et al.*, 2002).

ROLE OF INTEGRINS AND LECTINS IN MIGRATION OF T CELL AND DC SUBSETS

T cell subsets

Naïve T cells recirculate between the blood and the secondary lymphoid tissues. Upon encountering antigen, they loose the capability to home to lymph nodes and enter peripheral tissues (Hamann & Jablonski-Westrich, 1993). Activated and memory T cells have a higher potential to enter inflamed tissue than do naïve T cells. This might be due to the higher expression of adhesion molecules on activated and memory T cells when compared to naïve T cells (Hemler, 1990; Shimizu *et al.*, 1990; Shimizu *et al.*, 1991; Hamann & Jablonski-Westrich, 1993; Picker *et al.*, 1993). Within the memory population adhesion molecule expression is different. Whereas high levels of L-selectin are expressed by naïve and $CCR7^+$ central memory T cells, $CCR7^-$ effector memory T cells have lower amounts of L-selectin at their surface, but express higher levels of β1 and β2 integrins, as well as homing receptors as CLA (Sallusto *et al.*, 1999a).

A less distinct difference in migratory properties was demonstrated between $CD4^+$ and $CD8^+$ T cells. *In vitro* studies in collagen lattices showed that a significant percentage of $CD8^+$ cells slowed down with time, whereas $CD4^+$ cells had a more sustained migratory capacity for up to 12 h (Friedl *et al.*, 1994). $CD8^+$ T cells have different expression levels of adhesion molecules than $CD4^+$ T cells. As example, αM, αX, and α4 are preferentially expressed on $CD8^+$ cells, whereas L-selectin is preferentially expressed on $CD4^+$ cells. Although the expression of αL is similar on both $CD4^+$ and $CD8^+$ cells, the mean fluorescence intensity is higher on $CD8^+$ cells, as holds true for αM (Cavers *et al.*, 2002).

Within the $CD4^+$ cells, Th1 cells express higher levels of β2 integrins than Th2 cells, while the expression of β1 is comparable (Clissi *et al.*, 2000). However, some α chains linked to β1 integrins are differentially expressed (Colantonio *et al.*, 1999; Clissi *et al.*, 2000). The adhesion of Th1 cells seems, in contrast to Th2 cells, chemokine sensitive. In this way, Th1 and Th2 cells may differ in their adhesive response following chemokine stimulation (Clissi *et*

al., 2000). This indicates that besides integrin expression, also other factors such as chemokines and activating signals regulate the extravasation of T cell subsets.

DC subsets

The adhesion and migration of DCs critically depends on their maturation state. Though both immature and mature DCs bind to fibronectin, binding and in particular cell spreading of immature DCs is much stronger than for mature DCs (Brand *et al.*, 1998; De Vries *et al.*, 2003). In addition, immature, but not mature DCs adhere to immobilized E- and P-selectin under static and flow conditions (Pendl *et al.*, 2002). Migration studies with immature and mature murine DCs showed enhanced migration of DCs upon maturation (Gunzer *et al.*, 2000).

The origin of the DCs also determines their migratory properties. For instance, murine DCs derived from spleen have a higher initial migratory capacity than DCs isolated from the epidermis (Gunzer *et al.*, 2000). MDCs are also more motile cells than PDCs (de la Rosa *et al.*, 2003).

Adhesion molecules are differentially expressed on the distinct DC subsets. As described above for T cells, adhesion molecule expression is related to the migration pathway. Epidermal LCs express the skin-homing molecule CLA and the laminin receptor α6. Dermal DCs lack the expression of CLA, and lymph node DCs have no α6 expression (Price *et al.*, 1997; Strunk *et al.*, 1997; Ebner *et al.*, 1998). Another example is the upregulated expression of αLβ2 on LCs upon their migration to lymph nodes (Anjuere *et al.*, 1999).

SCOPE OF THIS THESIS

Nowadays DCs are used as cell-based vaccines in anti-tumor therapy. DCs are loaded with tumor-antigen, tumor lysates, are fused with tumor cells or transduced with tumor-derived RNA or viral vectors. The migration of DCs from the injection depot to the draining lymph node is essential to stimulate T cells to induce an effective immune response. The subsequent migration of these T cells to the tumor is a prerequisite to eradicate tumor cells. The aim of this thesis is to investigate the role of (novel) adhesion molecules in DC and T cell migration.

Several migration systems exist to study leukocyte migration. Whereas some of these systems, like transwell and Boyden assays only provide information about a population of cells, more sophisticated migration assays are coupled to time-lapse video systems to monitor the migratory behavior of individual cells. The cell recognition in these latter systems is based on contrast differences between cells and the substratum or on *a priori* morphology criteria. In chapter 2, we describe an Automated Cell Track System (ACTS) to quantify and track migration of multiple cells. The focus of the algorithm is only directed to the detection of the cell centroids in the successive images. As migrating cells continuously change their shape during their movement, the algorithm should be robust to the changing morphology. We used this system to investigate the migration of T cells as well as immature and mature DCs (chapters 2 and 3). Interestingly, a substantial difference in adhesion and migration between immature and mature DCs was demonstrated, which can be attributed to a difference in the activation status of the β1 integrin (chapter 4).

Recently, a novel DC-specific C-type II lectin, DC-SIGN was described which binds ICAM-3 (Geijtenbeek *et al.*, 2000a) and gp120 playing a role in HIV-biology (Geijtenbeek *et al.*, 2000b). Since ICAM-2 and ICAM-3 have a high homology and lectins are involved in rolling interactions, we examined whether ICAM-2/DC-SIGN interactions are involved in DC migration (chapter 5). We demonstrate that DC-SIGN binds ICAM-2 and mediates rolling interactions.

Mature DCs are very well equipped to stimulate T cells. MHC class I, MHC class II as well as the costimulatory molecules CD80 and CD86 are highly expressed on mature DCs. Moreover, mature DCs are characterized by the expression of the maturation marker CD83. The selective expression and upregulation of CD83 on mature DCs suggest a functional role of CD83 in immune responses. Indeed, circumstantial evidence for a biological role of CD83 is shown in literature. Virus-infected DCs express less CD83 and displayed a reduced capacity to initiate T cell proliferation (Kruse *et al.*, 2000b; Jenne *et al.*, 2000). In addition, human sera contain low levels of soluble CD83. In chapter 6, we used CD83-Fc to investigate the direct function of CD83. Interestingly, CD83-Fc binds to both immature and mature DCs and has a functional role in DC biology by regulating DC-mediated immune responses.

Chapter 2

An automated multi well cell system to study leukocyte migration

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ABSTRACT

Design of automated image processing systems to determine migration characteristics of individual cells is not trivial. Every test sample requires separate recording and the analysis of individual cell tracks in two- or three-dimensional migration systems by time-lapse microscopy is extremely laborious. Here, we describe a new Automated Cell Track System (ACTS). In addition to contrast differences, which are used by existing analysis systems, the ACTS algorithms recognize cells on the basis of morphological similarities in successive images and adapt to the continuous shape changes of individual cells during migration. The system facilitates simultaneous analysis of multiple cells and the measurement of multiple wells in one single experiment.

We validated the system studying HSB-2 T cell migration in standard 96-well microtiter plates coated with ICAM-1-Fc protein or control CD14-Fc protein. Migration of HSB-2 T cells on ICAM-1-Fc is LFA-1-mediated and both the number and the speed of migrating cells depend on the ICAM-1-Fc concentration.

We show that automated analysis of the migration data yields similar results as manual analysis, but in a fraction of the time. We conclude that this system is extremely well suited to precisely monitor the migratory behavior of individual cells. The analysis of multiple wells in parallel makes this set-up appropriate in high throughput screening in which multiple components are simultaneously tested for their effect on cell migration.

INTRODUCTION

Cell migration is essential in many immunological processes, such as inflammation, responses against tumors and viruses, and leukocyte homing. During these processes, dynamic interactions occur between cells and their environment, including other cells and extracellular matrix molecules (Huttenlocher *et al.*, 1995). Several well-established methods, such as the Transwell and Boyden assays, are used to study these interactions during migration (Wilkinson, 1982). In these assays, micropore filters are either coated with extracellular matrix molecules, cells, or other proteins of interest and the adhesion, as well as factors inducing migration, can be investigated. The migration is defined as the percentage of cells passing the filter. However, information about cell spreading, speed, or the migratory capacity of individual cells cannot be obtained from such assays (Wilkinson, 1982).

Therefore, two-dimensional and three-dimensional assays coupled to time-lapse video systems have been developed to monitor the migratory behavior of individual cells or of a population of cells in time (Thurston *et al.*, 1988; Friedl *et al.*, 1993; Niggemann *et al.*, 1997; Soll *et al.*, 2000; Demou & McIntire, 2002). Cells are either detected by light or fluorescence microscopy (Soll, 1988; Chon *et al.*, 1997; Chon *et al.*, 1998). These migration assays permit evaluation of the dynamics of the cells, such as distance of migration, migration speed, direction, and duration of migration. First, cells were tracked manually but more recently, automatic tracking programs have been generated to investigate their motility (Poole *et al.*, 1988; Bergman & Zygourakis, 1999; Demou & McIntire, 2002). Cell recognition is based on contrast differences between cells and the substratum or *a priori* morphology criteria and the focus of the software is directed to feature extraction of relevant cells (Soll, 1988; Wu *et al.*, 1995; De Hauwer *et al.*, 1999; Hoppe *et al.*, 1999).

In this report, we describe an Automated Cell Track System (ACTS) to quantify and track migration of multiple cells. The focus of the algorithm is directed to the detection of the cell centroids in the successive images. As migrating cells continuously change their shape during their movement, the algorithm should be robust to the changing morphology. Furthermore, multiple samples can be evaluated in this system by repetitive and simultaneously scanning multi-well plates, which is less time-consuming and will be more reproducible than systems in which every sample requires separate recordings in sequential experiments.

Using this tracking system, we studied the motility of HSB-2 T cells on ICAM-1-Fc (Intercellular Cell Adhesion Molecule-1-Fc chimera). HSB-2 T cells express the cell adhesion molecule LFA-1 (Leukocyte Function associated Antigen-1), which plays a pivotal role in adhesion as well as in migration processes. LFA-1 is a $\beta 2$ integrin, which binds ICAM-1 (Marlin & Springer, 1987) with high affinity. Other ligands of LFA-1 are ICAM-2 (Dustin *et al.*, 1986) and ICAM-3 (de Fougerolles *et al.*, 1993). For maximal binding, LFA-1 must be activated, which involves a conformational change of the molecule, leading to an increase of affinity and/or avidity for its ligands (Binnerts & van Kooyk, 1999).

We demonstrate that this ACTS is well suited to automatically track individual HSB-2 T cells migrating on ICAM-1-Fc in multiple wells.

MATERIAL AND METHODS

mAbs and Chemicals

Monoclonal antibodies reactive against different adhesion molecules were used: SPV-L7 (IgG1) (Keizer *et al.*, 1985), NKI-L15 (IgG2a) (Keizer *et al.*, 1985), and NKI-L16 (IgG2a) (Keizer *et al.*, 1988); a β2 blocking antibody AZN-L19 (IgG1) (Geijtenbeek *et al.*, 2000c), and the activating antibody KIM185 (IgG1) against the β chain of LFA-1 (Andrew *et al.*, 1993); KIM 225 (IgG1) against the α-chain of Mac-1 was a kind gift of Dr. M. Robinson (Celltech, Berkshire, UK); REK-1 (IgG1) reactive with ICAM-1 (Binnerts *et al.*, 1994). Other chemicals used were: deoxyglucose (50 mM, Sigma Chemical Co, St. Louis, MO), sodium azide (10 mM; Merck, Hohenbrunn, Germany) to deprive cells of energy, cytochalasin D (5 mg/ml, Sigma Chemical Co., St. Louis, MO) to disrupt integrin-cytoskeletal interactions, poly-L-lysine (Sigma Chemical Co., St. Louis, MO), and formaldehyde (3%; MERCK, Hohenbrunn, Germany).

Cells

The human T cell line HSB-2 (ATCC) was cultured in Iscove's medium (Gibco, Life Technologies ltd., Paisley, Scotland), containing 5% FCS and 1% Antibiotics/antimycotics (containing 100 U/ml penicillin, 100 mg/ml streptomycin, 0.25 mg/ml amphotericin B) (Gibco, Life Technologies ltd., Paisley, Scotland).

Immunofluorescence

Cells (50 x 10^3) were incubated with 2-5 μg/ml mAb (25 μl/well) in PBA (PBS containing 0.5% bovine serum albumin and 0.01% NaN_3) for 30 min at 4°C. After one wash step with cold PBA, cells were incubated with 25 μl FITC-labeled goat anti-mouse secondary antibodies (Zymed, San Francisco, CA; 1/50 dilution in PBA) for 30 min at 4°C. Subsequently, cells were washed and diluted in 100 μl PBA. Fluorescence was measured using a FACScan® (Becton and Dickinson & Co., Oxnard, Ca).

Adhesion Assay

96-well flat bottom Maxisorp plates (Nunc., Roskilde, Denmark) were coated with 50 μl goat anti-human Fc antibodies (Jackson Immunoresearch Laboratories, Inc., Westgrove, PA) at a concentration of 4 μg/ml in TSM (150 mM NaCl, 10 mM Tris/HCL, 2 mM MgCl2, 1 mM CaCl2, pH 8.0) for 60 min at 37°C, followed by blocking of the plate with 100 μl TSM/1%BSA. Subsequently, wells were coated with ICAM-1-Fc or CD14-Fc containing supernatant at the indicated concentrations O/N at 4°C as described previously (Binnerts *et al.*, 1996). Cells (7 x 10^6/ml) were fluorescently labeled with Calcein-AM (25 μg/ml; Molecular probes, Eugene, OR) for 30 min at 37°C and subsequently incubated (20,000-40,000/well) in the absence or presence of mAb, or stimulating or blocking agents on ICAM-1-Fc coated plates for 45 min at 37°C. Non-adherent cells were removed by gently washing steps with warm 0.5% BSA in TSM. Adherent cells were lysed with 100 μl lysis buffer (50mM Tris, 0.1% SDS) and their fluorescence (Calcein-AM) was quantified using the cytofluorometer (Perseptive Biosystems). Results are expressed as the mean percentage of adhesion of triplicate wells.

Migration assay

96-well flat bottom Maxisorp plates (Nunc., Roskilde, Denmark) were coated with ICAM-1-Fc or CD14-Fc as described in the adhesion assay or with poly-L-lysine. Cells (8 x 10^4/ml) were pre-incubated with either 5 μg/ml stimulating mAb, 80 μg/ml blocking antibodies, a combination of deoxyglucose (50 mM) and NaN_3 (10 mM), or with cytochalasin D (5 μg/ml) for 10 min at RT. Per well, 4,000 cells (50 μl) including the antibodies or reagents were added, resulting in 20 to 30 cells per image. Mineral oil (30 μl, Sigma Chemical Co., St. Louis, MO) was pipetted on top of the medium to prevent pH changes and evaporation of the medium. The coated plate with cells was placed on the microscope table and the experiment was started when the temperature of the medium in the control well had reached

37°C to start the experiment at physiological temperature, since LFA-1 binding critically depends on a physiological temperature (Figdor *et al.*, 1990), and to allow cells to settle to the bottom of the well. This was, in general, within 20 min. In the case of Poly-L-lysine coating, HSB-2 T cells (4,000/well) were seeded for 45 min, washed with PBS and fixed with 3.7% formaldehyde to validate hysteresis of the system.

Automated cell track system

The ACTS is depicted in Fig. 1A. A Zeiss (Thornwood, NY) Axiovert 35M inverted microscope equipped with the 10x/0.3 Ph1 bright phase contrast objective was connected to a 512 x 512 video camera (HCS MX5, DIFA, Breda, The Netherlands). Signals were digitized with Scion CG-7 or Pixel Pipeline frame grabbers in a Macintosh workstation (G4 or Quadra 800) (Boezeman *et al.*, 1997). The microscope was installed in a 37°C incubator to perform the experiments at physiologic temperatures. The wells of a 96-well plate were positioned in X, Y, and Z directions with an EK8b MTP scanning table (Marzhauser, Wetzlar, Germany) manually controlled by the operator or by the serial interface of the Macintosh computer during the experiment. The cycling moving table scanned a series of wells and sampled each well in parallel at a sample rate 1/30 Hz during at least 20 min. The images of each well were stored and linked to generate a movie.

Algorithm

The cells to be tracked were marked by the operator. A series of initial cell centroids $\{(x,y)_{grav,i}\ ,\ i=1,N\}$ was selected by pointing to the cell centers in the very first frame I_0 with typically N=8. The centroid $(x,y)_{grav}$ was calculated over all pixel values in the template:

$$x_{grav} = \Sigma(I_j.x_j) / \Sigma(I_j),\quad y_{grav} = \Sigma(I_j.y_j) / \Sigma(I_j)$$

The 32*32 pixel environment of each selected cell in the first frame I_0 initialized also the cell specific masks K_i. The algorithm then cycled through all successive frames I_j, j =1,J with three distinguished steps in each loop. First, the correlation function $U_{i,j}$ of mask K_i with the next frame j was calculated (Parker, 1997)(Russ, 1998)

$$U_{i,j} = I_j \bullet K_i$$

The position of the cell in the next frame was the weighted maximum of correlation function:

$$(x,y)_{i,j} = \max \{g(x_{i-1},y_{j-1},\tau), U_{i,j}\}$$

The weight function $g(x,y,\tau)$ benefits nearby maxima and prevents selection of a cell with similar morphology in the next frame. The scaling parameter τ is tuned with respect to sample frequency and cell speed. Lastly, the mask K_i was updated from the environment of the cell centroids in the new frame.

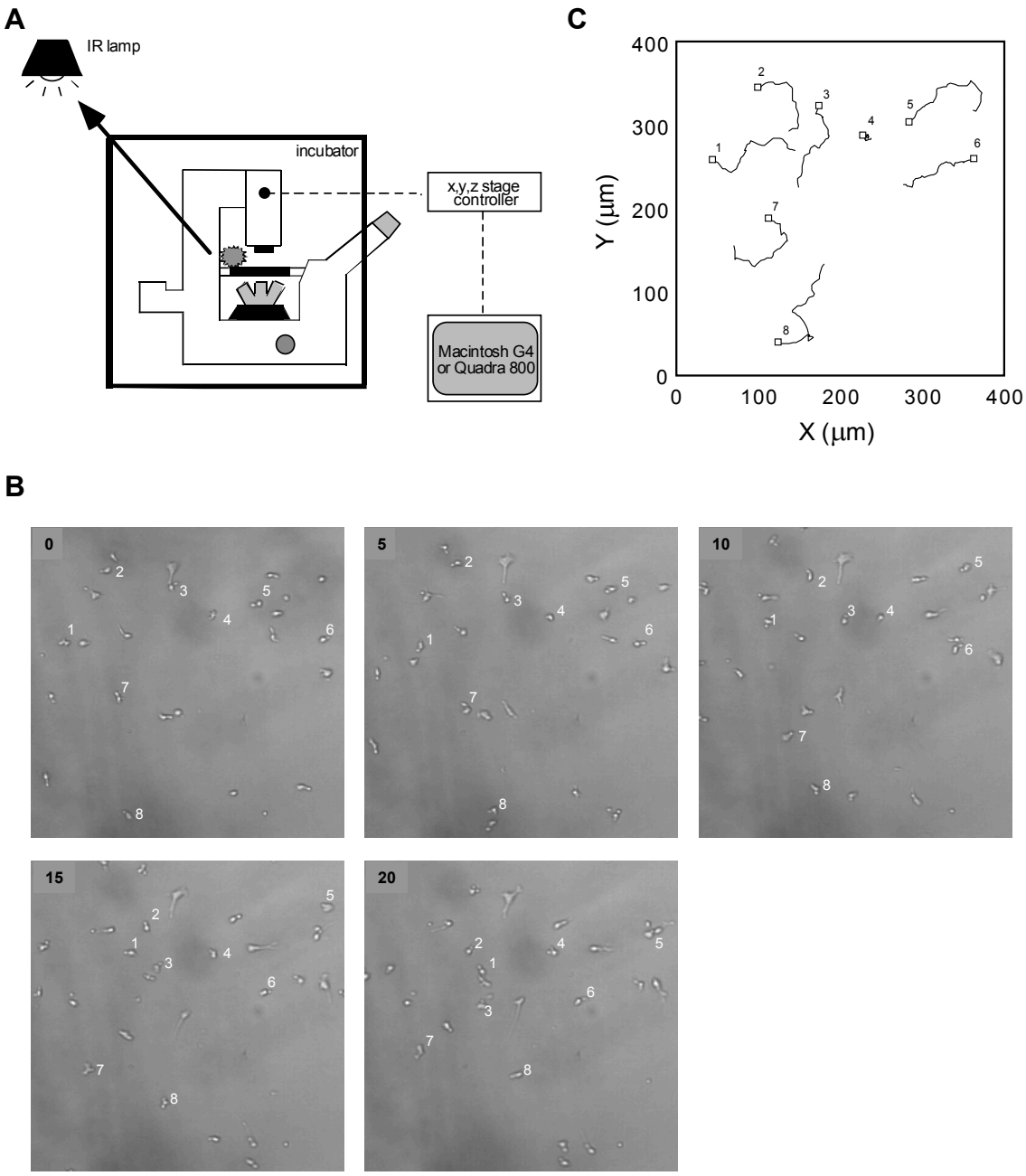

Figure 1. Schematic overview of the Automated Cell Track System (ACTS) and image, track analysis. (A) A 96-well plate is fixed onto an inverted microscope, which is connected to a video camera and coupled to a computer. The X, Y, and Z positions of the microscope table are regulated by a x, y, z stage controller. (B) Time-lapse recorded images of HSB-2 T cells. HSB-2 T cells (4,000/well) were seeded in an ICAM-1-Fc coated 96-well plate and monitored by the microscope every 30 s. Subsequently, the motility of the cells was analyzed with the tracking system. The images monitored at 0, 5, 10, 15, and 20 min are shown. (C) Traversed paths of the cells tracked in B. X and Y positions of the cells at each image are linked to generate the motility lines. Each square represents an individual cell at its starting position.

Data Analysis and Statistics

The tracked $(x,y)_{i,j}$ coordinates were visualized in time (Fig. 1B) with the most nearby pixels. Parameters, such as distance, speed, and direction were directly calculated from these coordinates, not from round-off pixel positions. Speed was calculated as the traversed path divided by the tracking

time. The traversed path of each individual cell was plotted in a graph as is shown in Fig. 1C. Cells that passed the image borders within the first 10 minutes of the experiment or which underwent cell division were excluded from the analysis.
Non parametric Kruskal-Wallis and Mann-Whitney tests were used to compare the reproducibility and specificity of different groups. Pearson correlation was applied to analyze the correlation between manual and automated tracking of the cells. Furthermore, Student's t-test was used to compare migration data from different experiments.

RESULTS

Adhesion molecule expression and adhesion profile of HSB-2 T cells

Cell migration is based on transient adhesive interactions both between cells and between cells and the extracellular matrix. Since β2 integrins are known to mediate leukocyte migration, we examined the β2 integrin expression of HSB-2 T cells and the adhesive properties mediated by these molecules, as a model. Flow cytometric analysis showed that HSB-2 T cells express high levels of the integrin β2 chain and the associated integrin unit αL (Leukocyte Function associated Antigen 1, LFA-1), but lack the expression of αM and αX (Mac-1 and p150,95 respectively) (Fig. 2A). Furthermore, LFA-1 is in an active state on these cells, since NKI-L16, an antibody reactive against the Ca^{2+} activation epitope, is expressed.
HSB-2 T cells spontaneously bind to ICAM-1-Fc coated plates (Fig. 2B), but not to CD14-Fc coated plates, which were used as controls (data not shown). The LFA-1-activating antibody KIM185, which causes a conformational change in LFA-1 (Andrew *et al.*, 1993), enhanced the adhesion of HSB-2 T cells to ICAM-1-Fc (Fig. 2B). It is important to realize that adhesion depends not only on the activation state of LFA-1, but also on integrin avidity and integrin distribution on the cell surface (Huttenlocher *et al.*, 1996; van Kooyk & Figdor, 2000). Adhesion to ICAM-1-Fc could be inhibited by blocking anti-LFA-1 antibodies, again indicating that adhesion of HSB-2 T cells to ICAM-1-Fc is LFA-1-mediated.

Effect of hysteresis

To evaluate the precision of the movement of the microscope stage, we plated HSB-2 T cells on poly-L-lysine, fixed the cells and measured the positions of the cells during continuously cycling and during stasis of the microscope stage (data not shown). In contrast to stasis conditions, cycling of the microscope stage induced a small mechanical jitter.
In addition, living cells change their morphology in successive images, which implies that the track dot placed within the cell at the start of the analysis moves continuously. This leads to false positives, cells that were identified as migrating, but instead, were non-migrating (cellular jitter). This phenomenon is illustrated in Fig. 3A and 3B, in which a non-migrating cell due to cellular jitter received an apparent motility by automated analysis. To reduce cellular jitter in our analysis we defined threshold distances, i.e. the distance the cell has to overcome in four subsequent frames (four 30 s intervals = 2 min in total). The x, y positions of a cell in frame I_j were included in the analysis when the displacement from frame I_j to frame I_{j+4} exceeded the minimal distance d. The reduction in cellular jitter was analyzed for increasing d values (d = 2, 4, or 6 μm) (Fig. 3B and 3D). Using these thresholds, motility of both visually scored migrating as well as non-migrating HSB-2 T cells coated on ICAM-1-Fc was determined (Fig. 3E and F). The cellular jitter was almost completely reduced by a d

value of 6 μm (Fig. 3E). This validation algorithm of x,y positions was applied to each cell in each frame in all migration experiments performed.
Next, we determined the percentage of migrating cells (in four experiments) using the track results (a migrating cell was defined as motility > 0.5 μm/min, based on results in Fig. 3E) and compared these percentages with visually scored migrating cells (Fig. 3G). Similar results (non-significant difference by Student's t-test) were observed, indicating that the track data discriminate very well between migrating and non-migrating cells, given the correction for cellular jitter.

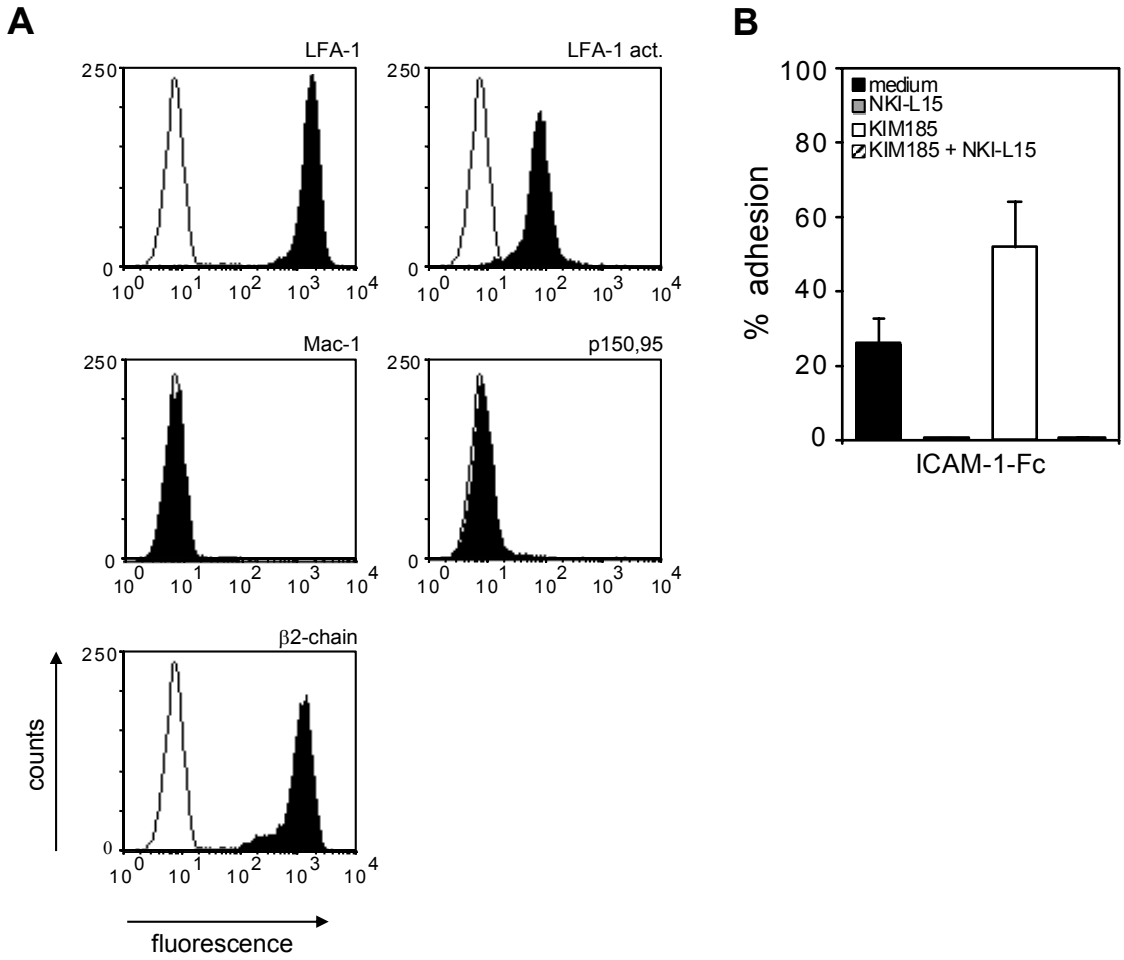

Figure 2. β2 integrin expression on HSB-2 T cells and LFA-1-mediated adhesion to ICAM-1-Fc. (A) Expression of β2 integrins on HSB-2 T cells by flow cytometry. HSB-2 T cells were stained for LFA-1 (NKI-L15), activation epitope of LFA-1 (NKI-L16), Mac-1 (KIM225), p150,95 (SHCL3), and β2 chain (NKI-L19). Open histograms represent the isotype controls, shaded histograms are the indicated antibodies. (B) Adhesion of HSB-2 T cells to ICAM-1-Fc. Activated HSB-2 T cells were incubated in wells coated with 50 ng/ml ICAM-1-Fc protein in the absence or presence of the LFA-1-activating antibody KIM185 (5μg/ml) and in the absence or presence of the LFA-1-blocking antibody NKI-L15 (10 μg/ml). Results are expressed as the mean % of adhesion of triplicate wells ± SD. Data are representative of three experiments.

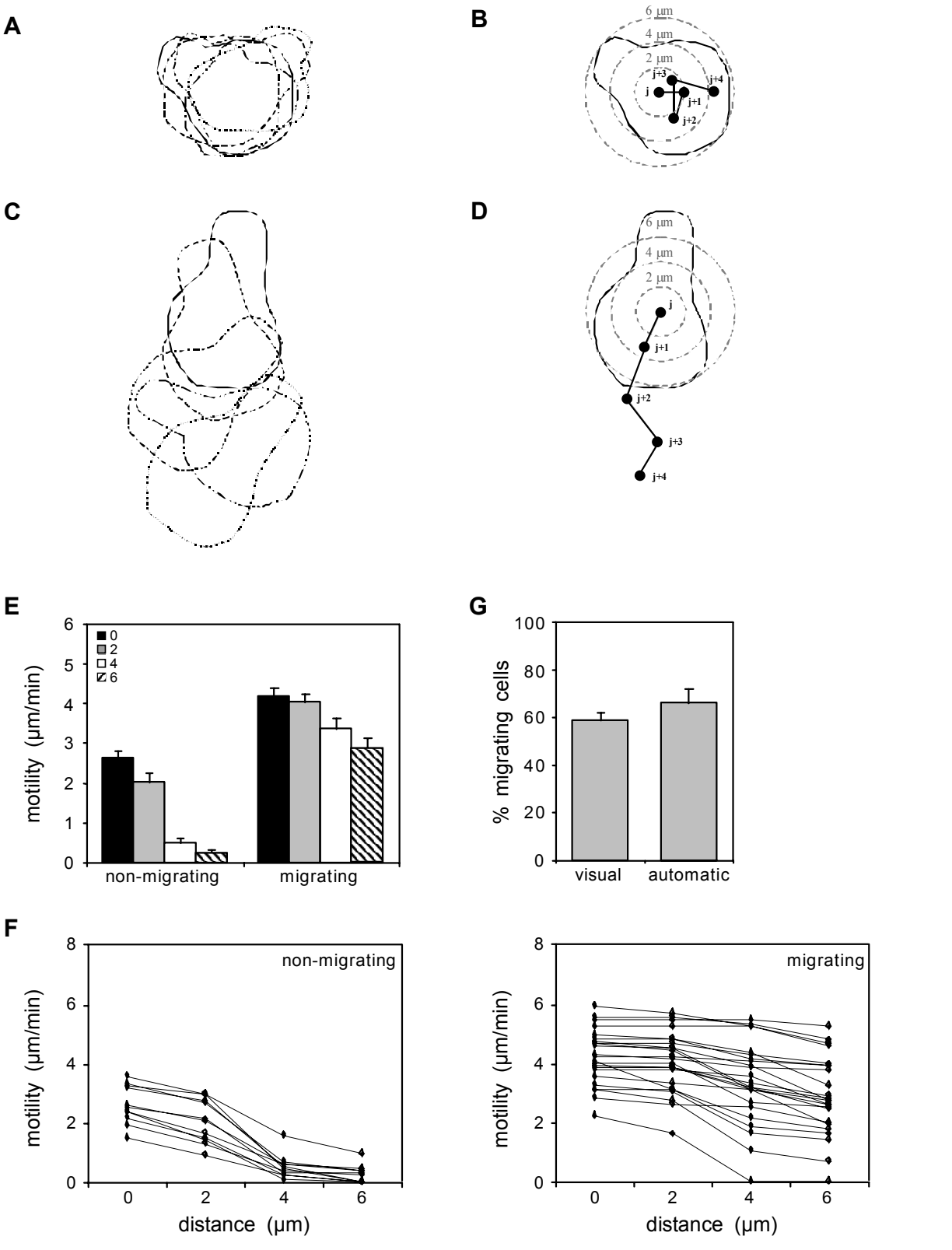
A
B
6 µm
4 µm
2 µm
j+3
j+4
j
j+1
j+2
C
D
6 µm
4 µm
2 µm
j
j+1
j+2
j+3
j+4
E
motility (µm/min)
0
2
4
6
non-migrating
migrating
G
% migrating cells
visual
automatic
F
non-migrating
migrating
motility (µm/min)
distance (µm)

Figure 3. Correction of motility of migrating and non-migrating cells. Illustration of a non-migrating (A) and a migrating (C) cell. Each overlay represents the position of the cell in a next frame. x, y positions and tracks during migration of the non-migrating (B) and migrating (D) cell. As illustrated, due to cellular jitter the automated track system falsely assigns distances to non-migrating cells (B). We introduced threshold distances (2, 4, and 6 μm) to distinguish migrating cells from cellular jitter (B and D). HSB-2 T cells were seeded on either 50 ng/ml ICAM-1-Fc or CD14-Fc (control coating). The wells were monitored every 30 s for a period of 20 min. Subsequently, cells were tracked and threshold distances were included to discriminate between migrating cells and non-migrating cells. As limits, 0, 2, 4, and 6 μm of displacement in 2 min were investigated. (E) Average motility of visually scored non-migrating and migrating cells at threshold levels of 0, 2, 4, and 6 μm. Motility is indicated in μm/min ± SEM. F. Speed of individual cells. Each square represents the speed of a cell after inclusion of the different threshold distances. G. Percentage of migrating cells determined visually and automatically (Motility < 0.5 μm/min is scored as non-migrating (decided on basis of speed in Fig. 3E). The mean ± SEM of four experiments is shown (Student's t-test, n.s.).

Validation of the tracking system

The automated tracking system was validated by comparison of the computer-assisted analysis with manual analysis of 20 cells by a blinded and a non-blinded observer. No significant differences were found between both observers (data not shown). Automated analysis has an advantage over manual analysis, since computer-assisted tracking is much less time-consuming (at least 10 times faster than manual analysis). Fig. 4 illustrates the excellent correlation (r = 0.98) between both analyses over a wide range of migration distances.

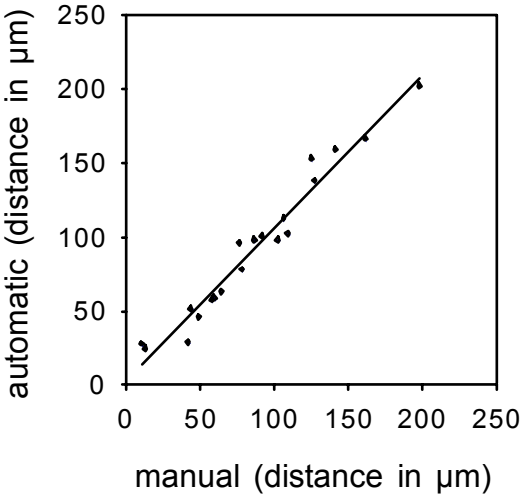

Figure 4. Comparison of migration distances of cells measured by manual as well as by automated tracking analysis. HSB-2 T cells were seeded on 50 ng/ml ICAM-1-Fc. The wells were monitored every 30 sec for a period of 20 min and subsequently tracked by manual and automated analysis. Distances are indicated in μm.

Reproducibility and specificity of the migration

HSB-2 T cells were used to investigate the reproducibility of our ACTS. The number of HSB-2 T cells that migrated on ICAM-1-Fc was measured in four separate wells. CD14-Fc was used as a control coating. As is depicted in Fig. 5A, cells were motile on ICAM-1-Fc (40±6% migrating cells), whereas on CD14-Fc they were considerably less motile (13±2% migrating cells, Mann Whitney U test $p<0.006$).

The software also permitted calculation of the speed of migrating cells. As is depicted in Fig. 5B, the average speed of the cells in four wells coated with ICAM-1-Fc was similar, i.e.

4.0±0.5 μm/min, 5.2±0.8 μm/min, 5.0±0.8 μm/min, 4.7±0.6 μm/min (non-significant, Kruskal Wallis). Differences in speed observed between individual cells reflect slower and faster moving cells. Taken together these results show that data obtained with this novel migration and tracking system are highly reproducible.
Substrate-ligand interactions together with an active cell metabolism and an intact cytoskeleton are a prerequisite for migration (Huttenlocher *et al.*, 1996). As HSB-2 T cells were motile on ICAM-1-Fc but not so on CD14-Fc, we studied the locomotion of HSB-2 T cells on ICAM-1-Fc in the presence of NaN_3 and deoxyglucose, and in the presence of cytochalasin D (Fig. 5C). In both cases, the motility of the cells was abolished, indicating that the locomotive behavior depended on metabolic energy and cytoskeletal rearrangements. Moreover, blocking antibodies against ICAM-1 (REK-1) and the β2 chain (NKI-L19) completely disrupted the motility on ICAM-1-Fc. Taken together, motility of HSB-2 T cells on ICAM-1-Fc is LFA-1-specific and depends on an intact cytoskeleton and an active cell metabolism.

Adhesion and migration are ligand concentration dependent

Both the integrin-mediated adhesion and the migration of cells on a substratum depend on ligand levels, integrin levels, integrin-ligand binding affinities, and integrin avidity (Huttenlocher *et al.*, 1996; Palecek *et al.*, 1997; van Kooyk & Figdor, 2000). To investigate the role of ligand concentration and integrin activity on HSB-2 T cell adhesion and migration, we studied both processes using 5, 50, and 500 ng/ml ICAM-1-Fc in the presence and absence of the LFA-1-activating antibody KIM185.
The adhesion of HSB-2 T cells was enhanced at increasing concentrations of ICAM-1-Fc and could be completely blocked by NKI-L15, demonstrating the specificity of the interaction. Furthermore, activation of LFA-1 by KIM185 antibodies enhanced cellular adhesion at a ligand concentration of 50 ng/ml, but no further increase of adhesion was observed at a concentration of 500 ng/ml ICAM-1-Fc (Fig. 6A), indicating that maximal adhesion was already reached at this extremely high ligand concentration.
The number of migrating HSB-2 T cells also depended on ICAM-1-Fc concentrations (Fig. 6B). At 50 ng/ml and 500 ng/ml ICAM-1-Fc, activation of LFA-1 by KIM185 completely blocked migration. The specificity of the interaction was confirmed using blocking antibodies. In contrast to the adhesion to ICAM-1-Fc that could not be further induced by KIM185 at 500 ng/ml (Fig. 6A), migration was further reduced as depicted in Fig. 6B. These apparent differences can be explained by the fact that the plate adhesion assay provides only a rough indication of adhesive strength (it measures only strong adhesion due to stringent washing), whereas the migration assay provides information on all cells including weakly adherent cells.
In addition, the speed of migrating cells at different ICAM-1-Fc concentrations was measured (Fig. 6C). The results showed that the speed of migrating cells was dependent on the ligand concentrations, exhibiting maximum levels at 50 ng/ml ICAM-1-Fc.
From these findings, we conclude that LFA-1-mediated adhesion as well as migration of HSB-2 T cells on ICAM-1-Fc depends on both the activation state of LFA-1 and the concentration of substrate coated.

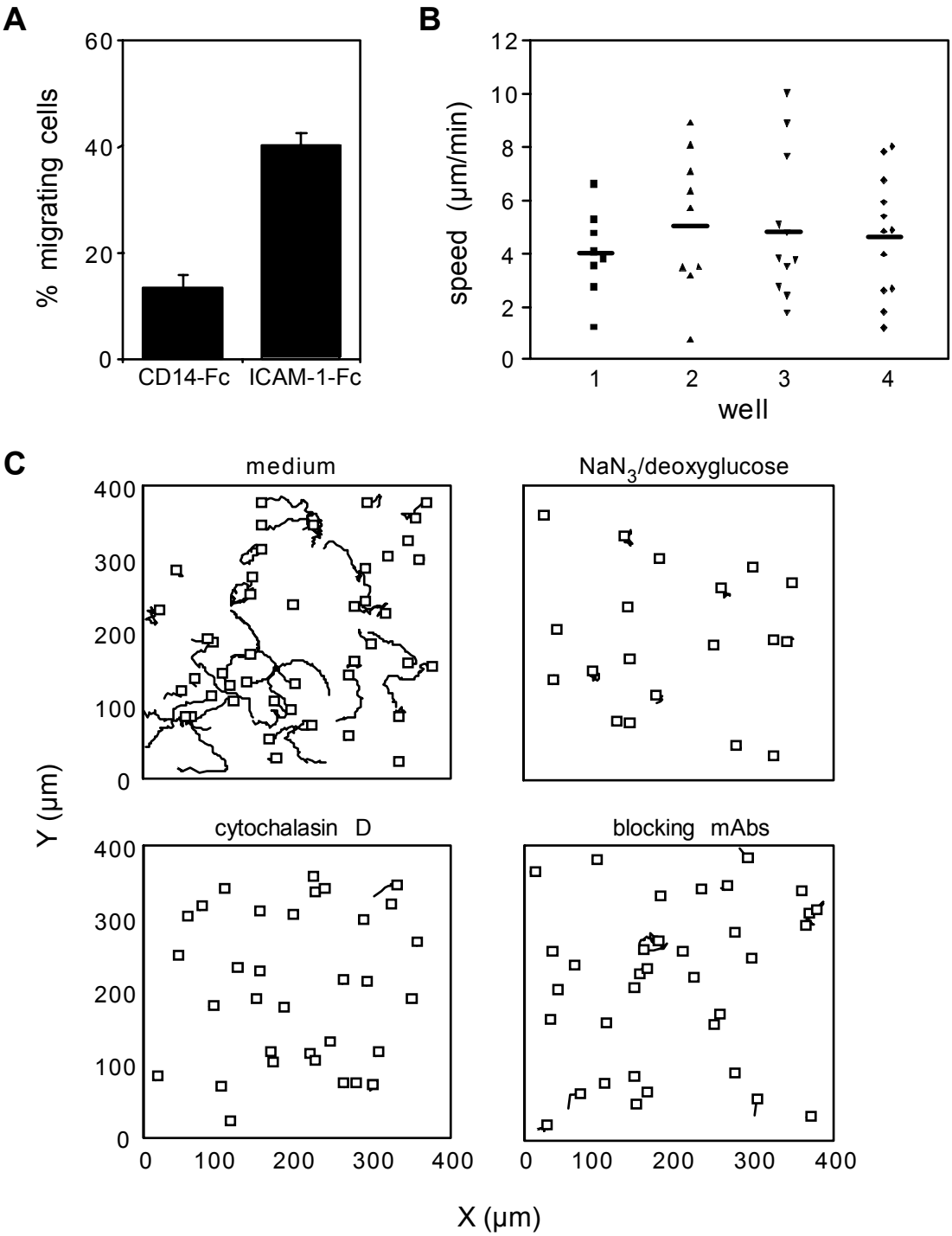

Figure 5. Reproducibility and specificity of the migration system. HSB-2 T cells in medium were seeded in four wells coated with either 50 ng/ml ICAM-1-Fc or CD14-Fc (control coating) (A and B), or with 30 ng/ml ICAM-1-Fc (C). The wells were monitored every 30 s for a period of 20 min (A and B) or 40 min (C). (A) Percentage of migrating cells on ICAM-1-Fc and CD14-Fc. The mean percentage of cells that migrate ± SEM in four different wells is presented. Data are representative of three experiments. (B) Average speed (in µm/min) of individual cells in four ICAM-1-Fc coated wells and overall average speed (-) in µm/min. Data are representative of three experiments. (C) Cell tracks of HSB-2 T cells in the absence or presence of NaN_3 and deoxyglucose, cytochalasin D, or a combination of ICAM-1-blocking (REK-1) and β2 chain-blocking (NKI-L19) antibodies. Each square represents an individual cell at its starting position. Data are representative of two experiments.

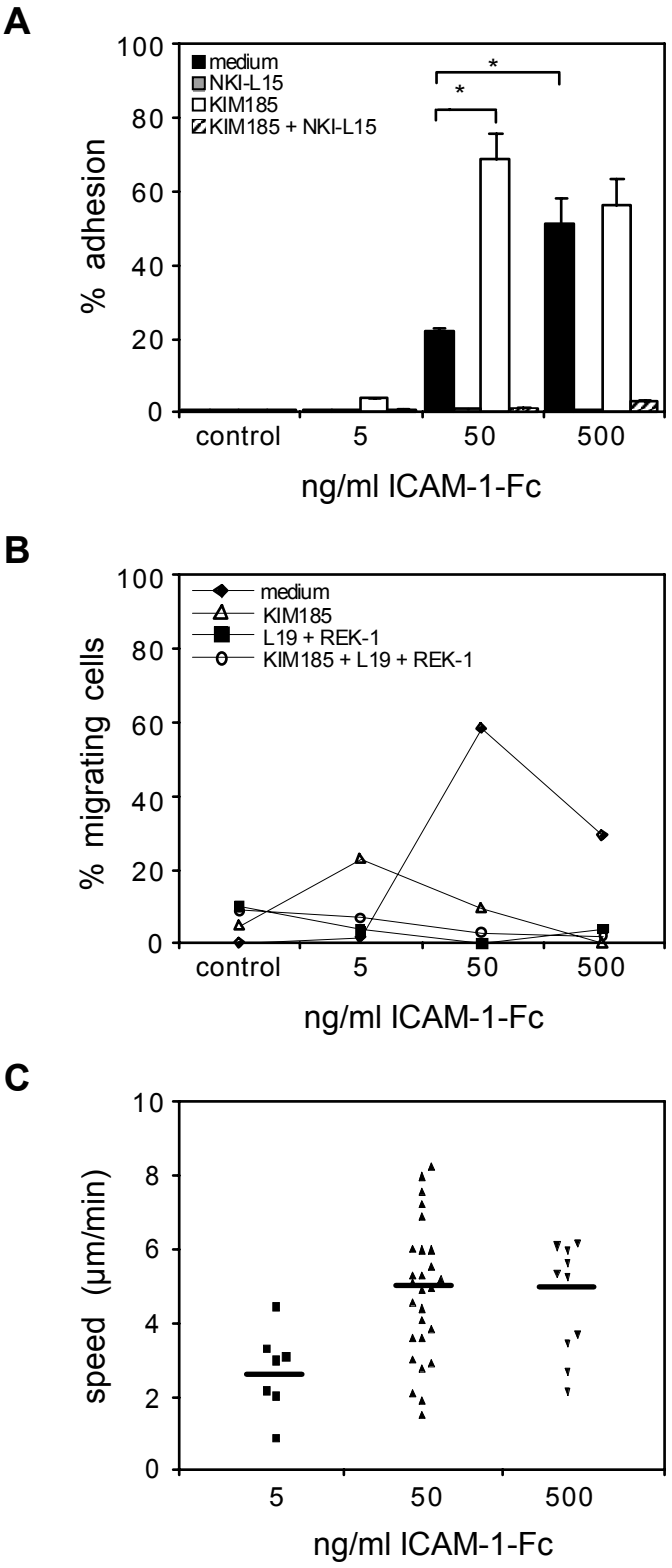

Figure 6. Adhesion and migration of HSB-2 T cells to different concentrations of ICAM-1-Fc. (A) Adhesion of HSB-2 T cells to ICAM-1-Fc. HSB-2 T cells were incubated on wells coated with a range of ICAM-1-Fc protein or with 50 ng/ml CD14-Fc as a control coating, in the absence or presence of the LFA-1-activating antibody KIM185 (5 μg/ml) and in the absence or presence of the LFA-1-blocking antibody NKI-L15 (10 μg/ml). Results are expressed as the mean % adhesion of triplicate wells ± SD. Data are representatives of three experiments (* $p<0.05$ in Mann Whitney U test). (B) Migration of HSB-2 T cells on ICAM-1-Fc. HSB-2 T cells were incubated on wells coated with a range of ICAM-1-Fc protein or with 50 ng/ml CD14-Fc (control coating), in the absence or presence of the LFA-1-activating antibody KIM185 (5 μg/ml) or with a combination of the ICAM-1-blocking antibody REK-1 (80 μg/ml) and the β2 chain-blocking antibody NKI-L19 (80 μg/ml). The wells were monitored every 30 s for a period of 20 min. Results are expressed as the % of migrating cells. Data are representatives of three experiments. (C) Average speed of migrating HSB-2 T cells on 5, 50, and 500 ng/ml ICAM-1-Fc. Speed of individual cells as well as the average speed (-) in μm/min is depicted. Data are representative of three experiments.

DISCUSSION

Several methods have been developed to study the migration of cells. Besides methods that only provide information on the number of migrating cells, such as the Boyden chamber and Transwell assay (Wilkinson, 1982), systems have been developed to study the migratory behavior of individual cells (Friedl *et al.*, 1993). Most of the techniques for automated cell tracking applied so far use contrast differences or fluorescent markers and *a priori* morphology criteria to recognize cells. Moreover, extraction of relevant cells is the main focus of the software of these systems (Soll, 1988; Wu *et al.*, 1995; Chon *et al.*, 1997; Hoppe *et al.*, 1999; Demou & McIntire, 2002). However, when the interface between cells and their environment is of low contrast or when two cells of equal contrast approach, the cells cannot be distinguished from each other and manual analysis is preferred. By contrast, the analysis performed in our system is based on the highly dynamic morphological alterations during cell migration (this is of particular relevance for lymphocytes, as shown in this report, but also for highly motile cells such as dendritic cells, as shown in the paper by (De Vries *et al.*, 2003)).

Typically 20 to 30 cells were recorded from each well. This sample size is representative for the population as shown by the high reproducibility of measurements in four subsequent wells. A further advantage of this newly designed imaging system is the possibility of performing multiple experiments under various experimental conditions in one series of recordings using only one set-up. In addition, the images are directly digitized into the workstation. Accordingly, our system is independent of the quality of videotapes and enables analysis without previously converting the data. Therefore, this multiple well migration system significantly reduces the workload (more than 10-fold reduction).

The accuracy of the tracking analysis is highly dependent on the speed of the cells and the density of the cell suspension. Our system records multiple wells in parallel by motion of the microscope table, which limits the sample rate. However, the sampling rate might be too low for fast migrating cells, since these cells have a large displacement per image. Hence, tracing of fast migrating cells will be more difficult and cells will easily be lost during analysis. Therefore, we reduced the time interval between individual images to 30 s in our experiments. Another feature of fast moving cells is that they induce a border problem, as they migrate out of the recorded area. As a consequence the tracking dot loses the cell near the edge of the image, which frequently results in jumping up and down along the recording border. Evidently, this results in incorrect migration data. Therefore, the software is designed such that it aborts tracking of a cell when it comes within a distance of 3 μm from the boundary.

As discussed above, the density of the cell suspension also affects cell tracking accuracy, the higher the cell concentration, the more difficult the tracking analysis. We found a density below 4,000 cells per well a satisfactory concentration to track the cells. In some cases, for instance when two approaching cells have highly similar morphology and contrast, the tracking system was not always able to discriminate between those cells. Although such events will not influence the overall results, the system allows the operator to interrupt the analysis to manually position the track dot back onto the cell after which the automated analysis can proceed.

Both the cycling of the microscope stage and the dynamics of the cells induced jitter in successive images of the same well. As a consequence cells scored as non-migrating

appeared to have an apparent speed between of 2.8±0.2 μm/min (see Fig. 3). Visual inspection of the time-lapse movies revealed that in most instances background speed resulted from a shift of the dot within the borders of the cell. Since the dot represents one pixel (1.52 μm) and the HSB-2 T cells have a diameter of approximately 10 μm this can lead to significant 'movement' of a cell. Therefore we defined migrating cells as cells exceeding a displacement of at least 6 μm in 2 min, since this threshold clearly reduced the speed of the non-migrating cells to background levels of nearly 0 μm/min.

Cell migration is a dynamic adhesive process, in which cells form new interactions with the substrate at their leading edge and detach from the substrate at the rear of the cell (Huttenlocher *et al.*, 1995). These adhesive forces can either be strong or weak, depending on the cell and substratum type. When the interaction is very weak or absent, as investigated with the plate adhesion assay, a synchrone flow of cells in the wells can be detected, despite the regulated temperature of the incubation chamber. A likely explanation for the flow of weak binders is the remaining liquid convection. Addition of mineral oil on top of the medium prevented not only pH changes but also evaporation of the medium thereby reducing this phenomenon. The direction of the movement of the stage was not correlated with the direction of the flow of the cells (data not shown), indicating that mechanical movement of the microscope stage did not contribute to this non-specific movement of non-, or weakly- binding cells.

To test the applicability and accuracy of this new migration system, we used the T cell line HSB-2 to study its adhesive and migratory capacities on ICAM-1-Fc. Since LFA-1 on HSB-2 T cells is already partially activated, the cells spontaneously bind to ICAM-1-Fc (Figdor *et al.*, 1990; van Kooyk *et al.*, 1994). However, further activation of LFA-1 with the LFA-1-activating antibody KIM185 results in an even higher percentage of bound HSB-2 T cells (Robinson *et al.*, 1992; Andrew *et al.*, 1993; Lub *et al.*, 1997).

Here, we demonstrate that HSB-2 T cells migrate on ICAM-1-Fc. This migration is LFA-1 specific, since blocking antibodies against LFA-1 and ICAM-1 could inhibit the motility to background levels. Moreover, activation of LFA-1 with KIM185 affected the migratory behavior of HSB-2 T cells on both 50 and 500 ng/ml ICAM-1-Fc. These data are in accordance with previous studies showing that CD2 activation (Carpen *et al.*, 1991), T Cell Receptor stimulation (Dustin *et al.*, 1997) and activation via PMA induce the activation state of LFA-1, and result in reduced motility of leukocytes on ICAM-1-Fc. KIM185 did not enhance the adhesion at 500 ng/ml ICAM-1-Fc, but migration was clearly affected. It should be stressed that the plate adhesion assay and the migration assay are completely different tests. In the adhesion assay a stringent washing step is applied which removes all non-adherent cells. It therefore only gives a rough indication of the adhesive strength of cells. In contrast, in the migration assay all cells are present because no washing forces are applied. Hence, it is highly likely that weakly binding cells are the ones that are, because of their limited adhesion, highly motile (in the adhesion assay these cells will, because of their low adhesion be washed away). In the migration assay these cells will stop migrating when activated with KIM185 and stick firmly, thus explaining the apparently different results between the adhesion and migration assays. This difference is also observed for control conditions (CD14-Fc), in which weakly binding cells might account for the background level of migration, i.e. 10-15% of the cells.

We showed that motility of HSB-2 T cells was clearly dependent on the concentration of ICAM-1-Fc. The proportion of migrating cells reached a maximum at 50 ng/ml. At lower

ligand concentrations, adhesion strength was too low to generate traction for migration. At higher concentrations the adhesive forces became too strong, resulting in immobile cells (DiMilla *et al.*, 1991). These results are in line with the observations of Palecek and colleagues, who also showed this biphasic pattern of migration for the integrins $\alpha5\beta1$ and $\alpha IIb\beta3$ on fibronectin and fibrinogen respectively (Palecek *et al.*, 1997). Furthermore, they showed that the migration speed of cells depends on several variables, including ligand levels, receptor density, and receptor-ligand binding affinities (Palecek *et al.*, 1997). Likewise, the mean speed of the HSB-2 T cells also depended on the ICAM-1-Fc concentration.
In conclusion, the ACTS we describe is well suited to study cell migration. The advantage over previously described methods is the automated track analysis based on contrast differences as well as on the actual cell morphology, and the simultaneous measurement of multiple wells in one single experiment. Therefore, this novel method will be useful to rapidly study the effects of multiple factors (ligands, chemokines, cytokines) influencing the migration of different cell types.

ACKNOWLEDGEMENTS

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Chapter 3

Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state

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ABSTRACT

Dendritic cells are the professional antigen-presenting cells of the immune system. To induce an effective immune response, these cells should not only express high levels of MHC and costimulatory molecules but also migrate into the lymph nodes to interact with naïve T cells. Here, we demonstrate that *in vitro*-generated mature, but not immature dendritic cells, efficiently migrate into the T-cell areas of lymph nodes of melanoma patients. This difference is confirmed by *in vitro* studies, in which immature dendritic cells are strongly adherent, whereas mature dendritic cells remain highly motile. Our present findings demonstrate that the ability of dendritic cells to mount a proper immune response correlates with their ability to migrate both *in vitro* and *in vivo*.

INTRODUCTION

The Dendritic Cell (DC) is a specialized antigen-presenting cell that can induce *de novo* antitumor responses in patients (Hsu *et al.*, 1996; Nestle *et al.*, 1998; Thurner *et al.*, 1999). Tissue-resident immature DCs take up antigen, followed by a complex maturation and activation process that is characterized by an up-regulation of antigen-presenting MHC molecules and costimulatory molecules, as well as a switch in their adhesion- and chemokine- receptor repertoire (Sallusto *et al.*, 1998; Mellman & Steinman, 2001). Although this process is only partially understood, it has become clear that these phenotypical changes allow DCs to migrate from peripheral tissues to the lymph nodes, in which they present processed antigens to resting T cells (Saeki *et al.*, 1999).
Recent studies in cancer patients, in which the efficacy of *in vitro*-generated DC vaccines are evaluated, show that mature DCs, but not immature DCs, induce an effective antitumor response (Jonuleit *et al.*, 2001; Dhodapkar *et al.*, 2001). The incapacity of immature DCs to induce an immune response is at least in part because of a low expression of antigen presenting- and costimulatory- molecules. In addition, monocyte-derived immature DCs lack CCR7, required for migration into the T cell areas of lymph nodes (Takayama *et al.*, 2001; Parlato *et al.*, 2001). Hence, immature DC generated *in vitro* may not colocalize and interact with naïve T cells in the lymph nodes, a prerequisite for the induction of an effective immune response.
Animal studies have provided direct evidence that DCs injected subcutaneously, but not intravenously, preferentially migrate to the draining lymph nodes to induce a measurable antitumor effect (Eggert *et al.*, 1999). Similarly, administration of radiolabeled DCs in humans demonstrates that the tissue distribution depends on the route of administration. DCs injected intravenously accumulate in the spleen and liver, whereas DC injected subcutaneously or intradermally migrate to the draining lymph nodes (Mackensen *et al.*, 1999; Morse *et al.*, 1999).
Studies in which DCs are directly injected intralymphatically show that DCs localize in the draining lymph node (Mackensen *et al.*, 1999), but do not inform whether the injected DCs reach the T-cell areas of the lymphoid organs. Here we compare the migratory capacity of DC vaccines *in vitro*, by measuring morphology and speed of individual cells, with the behavior of these cells *in vivo*. By radiolabeling of the DCs, we investigate the effects not only of the maturation state but also the route of administration on DC migration *in vivo*. Our findings demonstrate that the migration of DCs is highly dependent on their maturation state and suggest that injection of monocyte-derived mature DCs is superior to the injection of immature DC preparations.

MATERIALS AND METHODS

Antibodies and immunostaining

The following mAbs were used: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13), anti-CD80 (all Becton Dickinson, Mountain View, California), anti-CD83 (Beckman Coulter, Mijdrecht, The Netherlands), anti-CD86 and anti-CCR5 (both Pharmingen, San Diego, CA), anti-CCR7, AZN-D1 against DC-SIGN, HP2/1 against α4, SAM-1 against α5, AZN-L19 against β2 integrins, and AIIB2 against β1 integrins (Developmental Studies Hybridoma Bank, Iowa City, IA).

Preparation of DC

DCs were generated from peripheral blood mononuclear cells (PBMCs) as described previously (Thurner *et al.*, 1999; De Vries *et al.*, 2002). After leukapheresis, PBMCs were used for the generation of monocyte-conditioned medium (MCM), and plastic-adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker, Walkersville, MD) supplemented with 2% pooled human serum (Bloodbank Rivierenland, Nijmegen, The Netherlands), IL-4 (500 U/ml), and granulocyte macrophage colony stimulating factor (GM-CSF, 800 U/ml; both from Schering-Plough, International, Kenilworth, NJ) (Thurner *et al.*, 1999). After the addition of KLH (10 µg/ml) on day 3-4, immature DCs were harvested on day 6-7.

Mature DCs were cultured as immature DCs. Autologous MCM with prostaglandin E2 (PGE_2, 10 µg/ml; Pharmacia & Upjohn, Puurs, Belgium) and 10 ng/ml recombinant TNF-α (kindly provided by Dr. Adolf, Bender, Vienna, Austria) were added on day 7 (30%, v/v). Mature DCs were harvested on day 9.

Adhesion and migration assay

Flat-bottom plates (96-well; Costar, Corning, NY) were coated with 20 μg/ml fibronectin (Roche, Mannheim, Germany) and blocked with 0.01% gelatin (Sigma Chemical Co., St. Louis, MO). DCs (7 x 10^6/ml) were labeled with Calcein-AM (25 μg/ml; Molecular probes, Eugene, OR) for 30 min at 37°C, either untreated or preincubated (10 min, room temperature) with blocking mAb (10 μg/ml), and seeded on fibronectin-coated plates (20,000-40,000/well) for 45 min at 37°C. Non-adherent cells were removed by gently washing steps with warm 0.5% BSA (Boeringher Mannheim, Germany) in TSM (150 mM NaCl, 10 mM Tris/HCL, 2 mM MgCl2, 1 mM CaCl2, pH 8.0). Adherent cells were lysed with 100 μl of lysisbuffer (50mM Tris, 0.1% SDS) and fluorescence was quantified using the cytofluorometer (PerSeptive Biosystems). Results were expressed as the mean percentage of adhesion of triplicate wells.

We used our previously established migration assay to study migration of DCs. Four thousand DCs (40 μl) per well were seeded on fibronectin-coated plates, resulting in 100 cells per image. DCs were recorded for up to 100 min, after which, migration tracks of individual DCs were analyzed. The speed is defined as the traversed path during the entire experiment divided by the imaging time.

Patients

Melanoma patients, participating in an ongoing protocol in which the *in vivo* immune responses of a DC vaccine are studied (KUN 99-150) were included in this study. Eligibility criteria included stage III/IV melanoma, planned radical lymphadenectomy for lymph node metastases, HLA-A2.1 phenotype, melanoma expressing gp100 and tyrosinase, and written informed consent. The local regulatory committee approved the study. Twenty-four to 48 h before surgery, eligible patients received a single injection of the ^{111}In-labeled DCs ($10x10^6$, 200 μl saline) either intradermally in the proximity, or intranodally directly into a lymph node of the lymph node region that was to be resected. Intranodal injections were performed under ultrasound guidance.

At the same time, patients also received the DC vaccine (DCs pulsed with peptides gp100:154-162, gp100:280-288, and tyrosinase:369-377) intradermally or intranodally at a clinically tumor-free lymph node region. This was repeated for a total of four injections every 2 weeks.

After the DC vaccinations, a delayed type hypersensitivity (DTH) reaction was performed. Briefly, DCs (5 x 10^5 in 0.2 ml), either loaded with KLH and/or peptide or unloaded, were injected on the back by using a 25-gauge needle and a 1 ml syringe. The DTH was characterized by swelling, erythema, and induration. The diameter (in millimeters) of edema and induration was measured after 48 h.

Proliferative response to KLH

Cellular responses against the protein KLH were measured in a proliferation assay. Briefly, per well of a 96-well tissue culture microplate, $1x10^5$ PBMC, isolated from blood samples taken after one DC

vaccination, were plated either in the presence of KLH or without. After 4 days of culture, 1 μCi/well of tritiated thymidine was added, and incorporation of tritiated thymidine was measured in a beta-counter.

^{111}[In]oxinate labeling, administration and autoradiography

Immature and mature DCs were labeled with ^{111}indium oxinate (Mallinckrodt Medical, Petten, The Netherlands) in 0.1 M Tris-HCl (pH 7.0) for 15 min at room temperature as described previously (Eggert *et al.*, 1999; De Vries *et al.*, 2002). Scintigraphic images of the depot and corresponding lymph node basin were acquired with a gamma camera, 24 or 48 h after injection. After the last imaging session, the lymph node basin was resected. The radioactive lymph nodes in the resected specimen were identified and counted in a gamma counter in combination with injection standards. The fraction of ^{111}In-labeled DCs that migrated from the injection depot was determined as a measure of their migratory capacity *in vivo*. Radioactive lymph nodes were embedded in paraffin. Sections (4-μm) were dipped in LM1 photographic emulsion (Amersham, Buck, United Kingdom) and exposed for 2-3 weeks at 4°C. After exposure, the sections were developed and poststained with haematoxylin and eosin.

RESULTS

Characterization and potency of immature versus mature DCs when used as vaccines

Immature and mature DC populations exhibited their respective characteristic phenotype and morphology (Fig. 1A). Expression of adhesion molecules, both β1 and β2 integrins, slightly decreased after maturation (Fig. 1B, and data not shown). CCR5 was expressed by ~40% of the immature DCs but was absent on mature DCs (Fig. 1B). Conversely, immature DCs lacked expression of the homing receptor CCR7, whereas this molecule was present on ~80% of the mature DCs (Fig. 1B). We tested both immature and mature DCs for their capacity to induce a "*de novo*" immune response *in vivo*. In an ongoing study, melanoma patients received vaccinations either with immature (n=9) or mature (n=10) KLH-loaded DCs. We observed that only mature DCs, already after a single vaccination, induced a proliferative response against KLH in the peripheral blood of these patients (Fig. 1C). Moreover, DTH reactivity towards KLH-loaded DCs was observed after vaccination with mature DCs in all patients tested (n=9), whereas none of the patients (n=7) vaccinated with KLH-loaded immature DCs showed any DTH.

Adhesive and migratory properties of DCs to fibronectin

To examine whether differences in adhesive and migratory behavior between immature and mature DCs contribute to their efficacy as a vaccine, we investigated their capacity to bind to the extracellular matrix protein fibronectin. Although both types of DCs bind to fibronectin, binding and, in particular, cell spreading of immature DCs was much stronger than of mature DCs (Fig. 2A and B). DC adhesion could be blocked completely by antibodies against β1 integrins but was only partially inhibited by β2 integrin blocking antibodies (Fig. 2C).

Adhesion of immature DC to fibronectin was mediated through $\alpha5\beta1$ because blocking $\alpha5$, but not $\alpha4$, antibodies inhibited this type of adhesion (Fig. 2D).
Consistent with the enhanced spreading of immature DCs on fibronectin (Fig. 2A), virtually none of these cells migrated when followed in time-lapse cinematography (Fig. 2E and G). Although cell membranes of immature DCs were highly dynamic, and expanding, contracting, and changing shape continuously, no migration was observed. In contrast, mature DCs were highly motile on fibronectin (Fig. 2F and G), using their dendrites to continuously attach and detach from the substrate. As a consequence, the speed of mature DCs was 10 times higher than of immature DCs, 5.0 μm/min versus 0.5 μm/min (Fig. 2 H, Mann Whitney U test, $P<0.0001$).

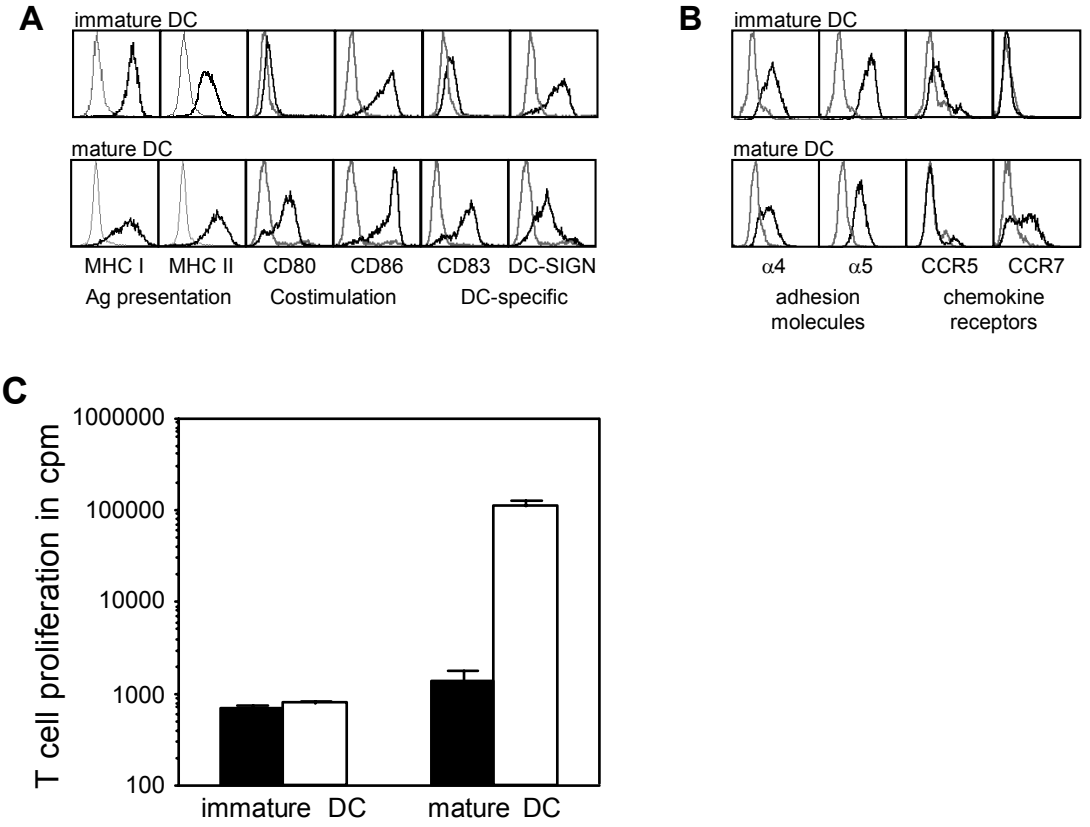

Figure 1. Characterization of immature and mature DCs. DCs were stained with mAbs against antigen-presentation molecules, costimulatory molecules, and DC-specific molecules (A), and adhesion molecules and chemokine receptors (B), and were measured by flow cytometry. Grey lines, isotype-matched controls; dark overlays, the markers as indicated underneath the histograms. Mature but not immature KLH-loaded DCs can induce an immune response *in vivo* after a single vaccination. (C) proliferative response against KLH in the PBMCs of the vaccinated patients was used as a readout: closed bars, without KLH; open bars, with KLH. The data from 1 of the 9 patients vaccinated with immature DCs and from 1 of the 10 patients vaccinated with mature DCs are shown.

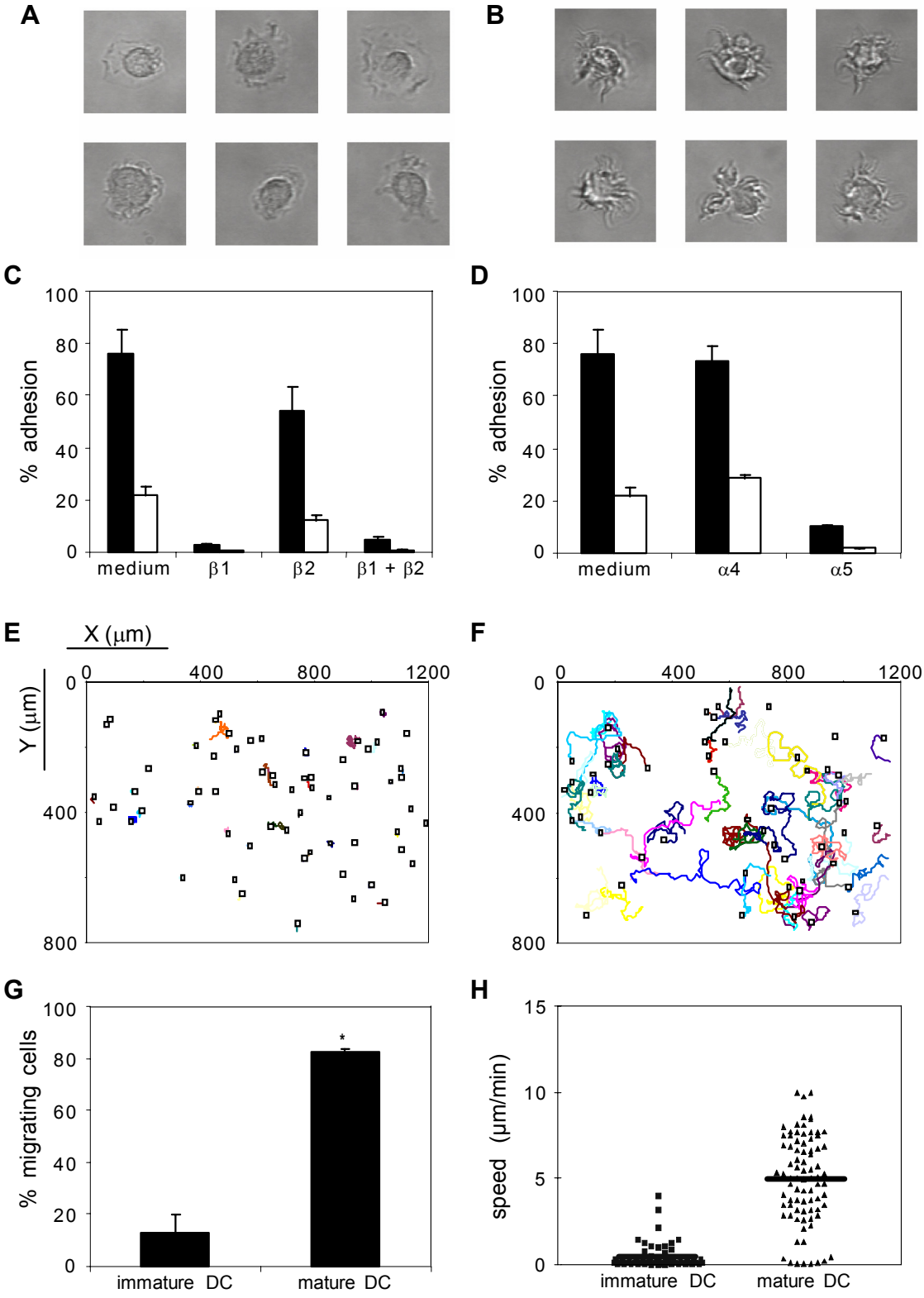
A
B
C
% adhesion
100
80
60
40
20
0
medium
β1
β2
β1 + β2
D
% adhesion
medium
α4
α5
E
X (μm)
Y (μm)
0
400
800
1200
F
G
% migrating cells
immature DC
mature DC
*
H
speed (μm/min)
15
10
5
immature DC
mature DC

Figure 2. Adhesion, spreading, and migration of DCs to fibronectin. Morphology of DCs on fibronectin-coated wells (A, immature DCs; B, mature DCs). Adhesion in the absence or presence of blocking antibodies against β1, β2 integrins (C), and α4, α5 integrins (D); closed bars, immature DCs; open bars, mature DCs. Results are expressed as the mean percentage of adhesion of triplicate wells. Data are representatives of three experiments. (E and F) different lines, migration paths of DCs: E, immature DCs; F, mature DCs. (G) percentage of migrated DCs ± SD of both immature and mature DCs (*, Wilcoxon-rank-sum test, P<0.01). (H) speed of migrated DCs ± SD (Mann-Whitney U test, P<0.0001); datapoints, the speeds of individual cells; horizontal bars, the mean speed. Data are representatives of three experiments.

Migratory capacity of DCs after injection into melanoma patients

To examine whether the differences in migration between immature and mature DCs were similar *in vivo* as observed *in vitro*, cells were labeled with radionucleotide ^{111}In (Eggert *et al.*, 1999; De Vries *et al.*, 2002). Previously, we showed that both immature and mature monocyte-derived DCs can be labeled with equal efficiency (95%), while remaining viable (~80%) for 24-48 h (De Vries *et al.*, 2002). Moreover, ^{111}In-labeling did not affect surface receptor expression by DCs (data not shown). *In vivo* migration of DCs was studied in melanoma patients, who received tumor-peptide pulsed DCs either intradermally (n=8 immature DCs; n=10 mature DCs) or intranodally (n=7 both immature and mature DCs), 24-48 h before radical dissection of regional lymph nodes. A time point between 24 and 48 h was chosen because the half-life of DCs, either unlabeled or ^{111}In-labeled, and of a MHC-peptide complex that they express, is limited (Kukutsch *et al.*, 2000).

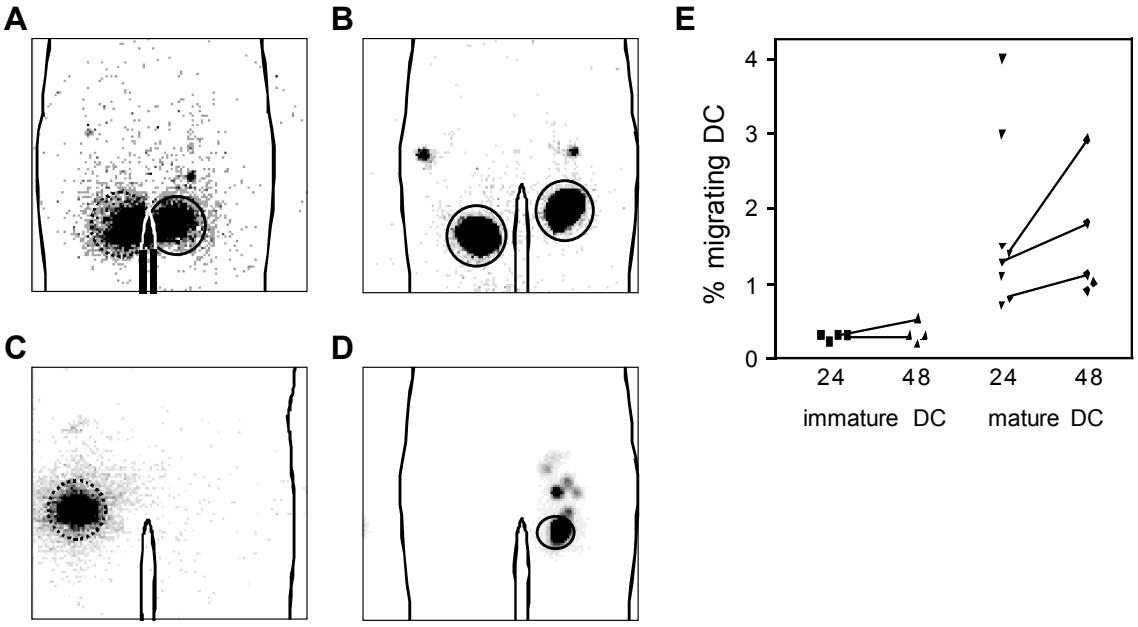

Figure 3. Biodistribution of DCs after injection. *In vivo* localization of intradermally (A and B) and intranodally injected (C and D) ^{111}In-labeled DCs by scintigraphic imaging with a gamma camera. Body contours are indicated. The four images are windowed differently to allow identification of the lymph nodes beyond the injection depot. (A) immature DCs 0.2%; mature DCs 0.8%. (B) mature DCs 1.5% and 1.1%. (C) 4%. (D) 23%. Dotted circles, injection depot of immature DCs. Closed circles, injection depot of mature DCs. ^{111}In-labeled DCs injected intradermally were measured for their migratory capacity *in vivo*. (E) Wilcoxon-rank-sum test, P<0.01; each symbol, one individual patient.

In accordance with previous results (Morse *et al.*, 1999), we observed that on intradermal injection, a significant percentage of both immature DCs and mature DCs remained at the site of injection (Fig. 3A and B). Nevertheless, a distinct amount of radioactivity was observed in the draining lymph nodes after injection of the DCs. Interestingly, a significantly higher percentage of mature DCs (mean ± SD, 1.8 ± 1.1%) migrated to lymph nodes and distributed over more lymph nodes (mean ± SD, 2.1 ± 1.6%) as compared to immature DCs (mean ± SD: 0.3 ± 0.1% migrated), which never migrated to more than one lymph node (Fig. 3A, B, and E). In addition, the migration of mature DCs, but not of immature, was somewhat enhanced after 48 h relative to 24 h. In one patient, we followed DC migration for up to 144 h, but no further increase in migration was observed.
Injection of DCs directly into a lymph node resulted in a large variation in the migratory capacity within the immature as well as the mature DC population. There was no difference (Wilcoxon rank-sum test) in migratory capacity of mature DCs (mean 19.3%; range 0.4% – 84%) versus immature DCs (mean 10.2%; range 0.5% – 30%) (Fig. 3C and D, and data not shown).

Intranodal localization of DCs after injection into melanoma patients

To investigate the capacity of DCs to migrate into lymph nodes, sections derived from resected lymph nodes distant from the node of injection were analyzed by microautoradiography. Explicit spots of radioactivity in the lymph node confirmed the migration of injected ^{111}In-labeled DCs into these nodes (Fig. 4). Intriguingly, a major difference between immature and mature DCs was observed. Immature DCs resided at the periphery of the nodes, the marginal sinus, whereas mature DCs migrated deeply into the T-cell areas further emphasizing their superior migratory properties (Fig. 4).

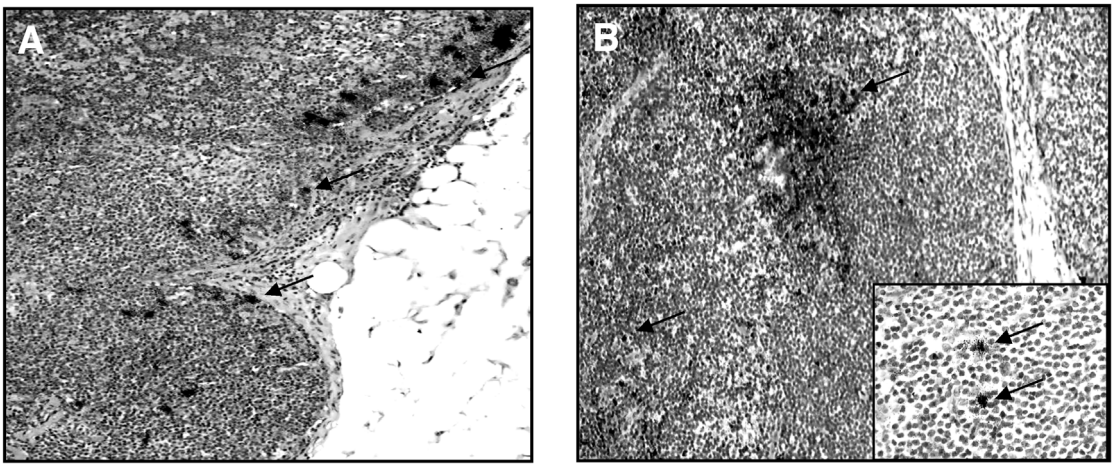

Figure 4. Distribution of intranodally injected ^{111}In-labeled DCs in distant lymph nodes *in situ*. After autoradiography, lymph node sections were stained with hematoxilin and eosin; black spots, the presence of ^{111}In-labeled DC (arrows). Overview of the lymph node section with immature DCs (A) and mature DCs (B).

DISCUSSION

Clinical studies in which tumor-antigen loaded DCs are used to vaccinate cancer patients indicate that mature DCs are superior to immature DCs to induce antitumor responses (Jonuleit *et al.*, 2001; Dhodapkar *et al.*, 2001). This might be attributed to their unique antigen processing and presentation machinery, which in mature DCs results in extremely high expression of antigenic peptides on MHC molecules and high levels of costimulatory molecules (Mellman & Steinman, 2001). However, next to their superior antigen-presenting properties, it is of utmost importance that antigen-loaded DCs come in close proximity of T cells in the lymph nodes (Cyster, 1999). Therefore, DCs injected into patients must actively migrate into the T-cell areas of lymph nodes. Here, we unequivocally demonstrate that mature DCs are migratory both *in vitro* and *in vivo*, irrespective whether they are administered into the skin or intranodally. By contrast, *in vitro*-generated immature DCs are unable to leave the skin after intradermal injection. Moreover, when migrating to distinct lymph nodes after direct lymph node application, immature DCs do not invade the T-cell areas, which precludes effective interactions with naïve T cells.

The low migratory capacity of immature DCs relates directly to their strong adhesive properties *in vitro*, which is mediated by highly expressed $\beta 1$ integrins, in particular $\alpha 5\beta 1$ (D'Amico *et al.*, 1998). Similar findings have been reported by Gunzer et al., who show, in a collagen matrix model, that the immature state of murine DCs is characterized by low migration, whereas mature murine DCs exhibit high migratory activity (Gunzer *et al.*, 2000). Our observations extend their findings by demonstrating that mature DCs weakly bind to extracellular matrix (fibronectin) and use their dendrites to actively migrate, whereas immature DCs stretch and flatten, thus hampering translocation.

Upon exposure to pathogens in peripheral tissues, resident DCs become activated through toll-like receptors (Kadowaki *et al.*, 2001), take up and process antigen, while migrating to the draining lymph nodes to present their antigenic load (Hirao *et al.*, 2000). To facilitate migration, CCR5 is down-regulated, while CCR7 is up-regulated, the latter being required for trafficking and entry into the T-cell areas of the lymph node (Dieu *et al.*, 1998; Sallusto *et al.*, 1998; Forster *et al.*, 1999; Saeki *et al.*, 1999; Gunn *et al.*, 1999). As expected, we observed that CCR7-positive mature DCs migrate into the T-cell areas of the lymph nodes, whereas immature CCR7-negative DCs do not. The inability of immature DCs to migrate into the T-cell areas could explain why vaccination with KLH-loaded immature DCs in melanoma patients fails to induce both proliferative as well as DTH responses. Our observations that immature DCs fail to induce an immune response correlates with studies of others. Immature DCs, when used as a vaccine adjuvant, might give rise to T cells that display the typical properties of regulatory T cells; non-proliferative, IL-10-producing T cells that can dampen pre-existing antigen-specific effector T-cell function (Jonuleit *et al.*, 2000; Dhodapkar *et al.*, 2001).

Previously, we and others showed that the majority of injected DCs reside at the injection depot (Mackensen *et al.*, 1999; Eggert *et al.*, 1999; Morse *et al.*, 1999; Barratt-Boyes *et al.*, 2000). Here, we demonstrate that monocyte-derived DCs that are matured *in vitro*, for the greater part, remain in the injection depot, although they express CCR7 and are highly motile *in vitro*. Gunzer et al. reasoned that the microenvironment largely influences emigration (Gunzer *et al.*, 2000). In addition, the relative high local density of DCs at the site of injection may affect this microenvironment, thereby having a major impact on emigration.

A better understanding of this lack of emigration, which can be enhanced with matrix metalloproteinases, is of importance for future DC vaccine development (Ratzinger *et al.*, 2002).
Although intradermally injected DCs migrate out of the skin very inefficiently, nevertheless, a 10-fold higher number of mature DCs migrated to an adjacent lymph node when compared to their immature counterparts. In addition, mature, but not immature, DCs migrate to multiple lymph nodes after intradermal injection. Intranodal application of immature or mature DCs leads to a substantial migration to several distant lymph nodes, as soon as 1 h after vaccination. Although only the mature DCs reach the T-cell areas, after intranodal injection of immature and mature DCs, little or no difference is observed between the migration to lymph nodes. Intranodal injection resulted in a rather variable migration in both cell populations. This might be because injection of DCs directly into a lymph node leads to a partial destruction of the lymph node architecture, resulting in the migration to distant lymph nodes, of DCs that would otherwise reside in the injected node. Likewise, during intranodal administration, a significant proportion of DCs may be transported by the flow of lymphatic vessels to nearby lymph nodes. A major advantage of intranodal over intradermal vaccination is, therefore, that an increased number of DCs are getting to the lymph nodes, e.g., the site at which the initiation of the immune response occurs. Especially when peptide-loaded DCs are used as a vaccine, the time required to reach the T-cell areas of the lymph nodes is of great importance, because the half-life of DCs and of a MHC-peptide complex that they express is limited (Kukutsch *et al.*, 2000). In the first clinical study in melanoma patients, which reported on the efficacy of peptide-loaded DC vaccines, mature DCs were injected intranodally. Our results may provide a mechanistic explanation for the effectiveness of the protocol used in that study (Nestle *et al.*, 1998).
Given the previously described tolerizing capacity of immature DCs, the recent results from mature-DC vaccination studies, and the migration data presented in this study, we conclude that mature DCs are preferred over immature DCs in clinical vaccination studies in cancer patients.

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Chapter 4

The activation state of the β1 integrin dictates adhesive and migratory properties of immature and mature dendritic cells to fibronectin

Daniëlle Krooshoop, Frank van Leeuwen, Karin Broers, Reinier Raymakers, and Carl Figdor

ABSTRACT

The capacity of DCs to activate naïve T cells depends on their maturation state and the migration to T-cell areas of the draining lymph nodes. Whereas mature DCs are highly migratory, immature DCs are strongly adherent. In the present study we investigated whether and how integrin expression contributes to these distinct adhesive capacities.
Although both immature and mature DCs express comparable levels of the integrin subunit β1, the adhesion of mature DCs is clearly reduced. A β1-activating antibody could restore this decreased adhesion, suggesting that the β1 integrin is less active on mature DCs relative to immature DCs. Indeed, the expression of active β1, as measured by the 12G10 antibody, is reduced on mature DCs. Upon fibronectin adhesion immature DCs, but not mature DCs, formed podosome structures containing active β1.
Together these findings imply that the adhesion of DCs to fibronectin is not regulated by expression levels, but rather by differential regulation of integrin activity during DC maturation.

INTRODUCTION

Dendritic Cells (DCs) are highly motile, professional antigen presenting cells derived from hematopoietic bone marrow stem cells (Banchereau & Steinman, 1998; Liu, 2001). DC precursors migrate via the blood into peripheral tissues, where they reside as immature DCs patrolling for antigen. Antigen uptake results in a change in DC function from an immature, antigen uptake phenotype into a mature, migratory, antigen presenting phenotype (Steinman, 1991; Banchereau & Steinman, 1998). This maturation process is characterized by the upregulation of MHC molecules, costimulatory molecules, but also by a switch in chemokine receptor expression from CCR1 and CCR5 to CCR4, CXCR4, and CCR7 (Sallusto *et al.*, 2000). CCR7 drives the migration of DCs from the skin into T cell areas of draining lymph nodes, where the mature DCs interact with naïve T cells to induce a primary immune response (Forster *et al.*, 1999).
During their migration from peripheral tissues to afferent lymphatics, DCs interact with extracellular matrix (ECM) molecules. Cell adhesion to ECM is regulated by special cellular adhesion structures, such as focal adhesions and podosomes. Focal adhesions are large adhesion contacts at the end of stress fibers (reviewed in Petit & Thiery, 2000). Podosomes, the most prominent adhesive organelles in DCs are short-lived structures that are formed upon cell-substrate contact (Marchisio *et al.*, 1988; reviewed in Linder & Aepfelbacher, 2003). Podosomes are related to focal adhesions and contain similar proteins, like actin, vinculin, talin, and integrins (Zamir *et al.*, 2000). The importance of podosomes for adhesion is underscored by podosome-deficient WAS macrophages that exhibit a defective adhesion (Linder *et al.*, 1999; Burns *et al.*, 2001).
β1 integrins are the main adhesion molecules implicated in cell-ECM interactions. Ligand binding depends on the activation state of the β1 integrin, which can be achieved via inside-out signaling. This activation process can be induced by signaling via other surface receptors, such as G-protein-linked receptors, or via phorbol esters that activate PKC and results in regulated clustering of integrins and subsequent adhesion strengthening (Grabovsky *et al.*, 2000; Stewart *et al.*, 1998). Moreover, β1 integrin-mediated adhesion can be induced from the outside of the cell by divalent cations, like Ca^{2+}, Mg^{2+}, and Mn^{2+}, or by activating antibodies (Gailit & Ruoslahti, 1988; van de Wiel-van Kemenade *et al.*, 1992; Arroyo *et al.*, 1992; Mould *et al.*, 1995).
Human epidermal Langerhans Cells (LCs) and human tonsillar follicular DCs adhere to fibronectin and laminin via $\alpha5\beta1$ and $\alpha6\beta1$ respectively (Le Varlet *et al.*, 1992; Ogata *et al.*, 1996). Upon antigen stimulation LCs upregulate $\alpha4\beta1$ expression, suggesting a role for this integrin in LC migration from the dermis into regional lymph nodes (Aiba *et al.*, 1993). The adhesion of human monocyte-derived DCs to a natural ECM ligand derived by endothelium is mediated by the β1 integrins $\alpha4\beta1$ and $\alpha5\beta1$ (D'Amico *et al.*, 1998; Bianchi *et al.*, 2000). We and others have shown that DC adhesion to fibronectin is mediated by $\alpha5\beta1$ (D'Amico *et al.*, 1998; Jancic *et al.*, 1998; De Vries *et al.*, 2003).
The adhesive and migratory properties of DCs critically depend on their maturation state. In a collagen matrix model, murine mature DCs are fast migrating cells, whereas immature DCs have slow migration characteristics (Gunzer *et al.*, 2000). Consistent with these observations human monocyte-derived mature DCs are highly migratory in comparison to their immature counterparts, both *in vitro* and *in vivo* (De Vries *et al.*, 2003).

We have previously shown that immature DCs more strongly adhere to fibronectin when compared to mature DCs. This difference in adhesive capacity correlated with a higher migratory capacity of mature DCs on fibronectin. In order to determine the mechanism responsible for these differences in adhesive potential, we have compared both expression levels and activity of β1 in immature versus mature DCs. We demonstrate that the increased adhesive capacity of immature DCs can be attributed to a higher expression of active β1 on immature DCs than on mature DCs. Moreover immature DCs form podosomes containing active β1 integrins, whereas these structures are absent in mature DCs. We conclude that the presence of podosomes is inversely correlated with a migratory DC phenotype.

MATERIALS AND METHODS

Chemicals and antibodies

The following mAbs were used: anti-HLA class I (W6/32), anti-HLA DR/DP (Q5/13), anti-CD80 (all Becton Dickinson, Mountain View, California), anti-CD14 and anti-CD83 (both Beckman Coulter, Mijdrecht, The Netherlands), anti-CD86 (Pharmingen, San Diego, California), AZN-D2 against DC-SIGN (Geijtenbeek *et al.*, 2000a), TS2/16 against ß1 integrins (Hemler *et al.*, 1984), AIIB2 against ß1 integrins (Developmental Studies Hybridoma Bank, Iowa City), 12G10 against active ß1 integrins (Serotec, Oxford, United Kingdom), anti-vinculin (Sigma, St. Louis, MO), isotype controls mIgG1 (Becton Dickinson, Mountain View, California), mIgG2a, mIgG2b, rIgG1 (all Pharmingen, San Diego, California), and the secondary antibodies goat anti-mouse Alexa-488 (Molecular Probes, Eugene, OR), goat anti-mouse-FITC (Zymed, San Francisco, CA), and goat anti-rat-PE (Becton Dickinson, Mountain View, CA). Rhodamin-conjugated phalloidin (Molecular Probes, Eugene, OR) was used to stain actin.

Preparation of DCs

DCs were generated from peripheral blood mononuclear cells (PBMCs) as described previously (Thurner *et al.*, 1999; De Vries *et al.*, 2002). Monocytes were derived from buffy coats or from a leukapheresis product. Plastic-adherent monocytes were cultured in X-VIVO 15 medium (BioWhittaker, Walkersville, Maryland) supplemented with 2% pooled human serum (PAA laboratories, Linz, Austria), IL-4 (500 U/ml) and granulocyte macrophage colony stimulating factor (GM-CSF, 800 U/ml) (both from Schering-Plough, International, Kenilworth, New Jersey)(Mackensen *et al.*, 1999). Immature DCs were harvested on day 7.

DCs were matured with monocyte-conditioned medium (MCM) with 10 ng/ml recombinant TNF-α (kindly provided by Dr. Adolf, Bender, Vienna, Austria) and prostaglandin E_2 (PGE_2, 10 µg/ml, Pharmacia & Upjohn, Puurs, Belgium) added on day 7 (30%, v/v). Mature DCs were harvested on day 9.

Immunofluorescence

Cells ($50x10^3$) were incubated with 2-5 µg/ml mAb (25 µl/well) in PBA (PBS containing 0.5% bovine serum albumin and 0.01% NaN_3) for 30 min at 4°C. After one wash step with cold PBA, cells were incubated with 25 ml FITC-labeled goat anti-mouse secondary antibody or goat anti-rat secondary antibody for 30 min at 4°C. Subsequently, cells were washed and diluted in 100 µl PBA. The fluorescence was measured using a FACScan® (Becton and Dickinson & Co., Oxnard, Ca).

Fluorescence microscopy

DCs were seeded on fibronectin coated glass coverslips (blocked with 0.01% gelatin) for 1 or 2 h and fixed in 3.7% formaldehyde/PBS for 10 min. Cells were permeabilized in 0.1% Triton X-100 and blocked with 2% bovine serum albumin in PBS. Cells were incubated with anti-vinculin antibody.

Subsequently, cells were stained with goat anti-mouse Alexa Fluor 488 and rhodamine-conjugated phalloidin. Images were collected by confocal laser scanning microscopy (Biorad) or fluorescence microscopy (Leica).

Adhesion assay

96-wells flat bottom plates (Costar, Corning, New York) were coated with 20 μg/ml fibronectin (Roche, Mannheim, Germany) for 60 min at 37°C, washed, and blocked with 0.01% gelatin (Sigma Chemical Co., St. Louis, Missouri) for 30 min at 37°C. DCs ($1x10^7$/ml) were labeled with Calcein-AM (25 μg/ml; Molecular probes, Eugene, OR) for 30 min at 37°C, either untreated or preincubated (10 min, RT) with blocking mAb (10 μg/ml), and seeded on fibronectin-coated plates (20-40,000/well) for 45 min at 37°C in the presence or absence of an activating antibody (1 μg/ml). Non-adherent cells were removed by gently washing steps with warm (37°C) 0.5% Bovine Serum Albumin (Boehringer Mannheim, Germany) in TSM (150 mM NaCl, 10 mM Tris/HCL, 2 mM MgCl2, 1 mM CaCl2, pH 8.0). Adherent cells were lysed with 100 μl lysisbuffer (50mM Tris, 0.1% SDS) and fluorescence was quantified using the cytofluorometer (PerSeptive Biosystems). Results are expressed as the mean percentage of adhesion of triplicate wells.

Migration assay

96-wells flat bottom plates (Costar, Corning, New York) were coated with 20 μg/ml fibronectin (Roche, Mannheim, Germany) and blocked with 0.01% gelatin (Sigma Chemical Co., St. Louis, Missouri) as described in the adhesion assay section. Per well, 3,000 cells (50 μl) were added resulting in approximately 100 cells per image. Mineral oil (30 μl, Sigma Chemical Co., St. Louis, MO) was pipetted on top of the medium to prevent pH changes and evaporation of the medium.
DCs were monitored with our previously described migration system (Krooshoop *et al.*, 2003). In brief, a Zeiss (Thornwood, NY) Axiovert 35M inverted microscope equipped with the 10x/0.3 Ph1 bright phase contrast objective is connected to a 512 x 512 video camera (HCS MX5, DIFA, Breda, The Netherlands). Signals were digitized with Scion CG-7 or Pixel Pipeline frame grabbers in a Macintosh workstation (G4 or Quadra 800)(Boezeman *et al.*, 1997). The microscope was installed in a 37°C incubator to perform the experiments under physiologic temperatures. The wells of a 96 well plate were positioned in X, Y, and Z directions with an EK8b MTP scanning table (Marzhauser, Wetzlar, Germany) manually controlled by the operator or by the serial interface of the Mac during the experiment. The repetitive cycling table scans a series of wells and samples each well in parallel at a sample rate 1/30Hz. DCs were recorded for up to 60 min followed by analysis of individual DCs. The speed was defined as traversed path during the entire experiment divided by the imaging time.

RESULTS

Characterization of immature and mature DCs

Human DCs were generated from monocytes and characterized by flow cytometry. Immature DCs expressed MHC class I and II, the costimulatory molecule CD86, the DC-specific marker DC-SIGN, expressed low levels of the costimulatory molecule CD80, and lacked the expression of the maturation marker CD83 (Fig. 1). Maturation of DCs by a combination of MCM, TNFα, and PGE_2 resulted in the induction of CD80 and CD83, an upregulation of MHC class I, MHC class II, and CD86, and a downmodulation of DC-SIGN.

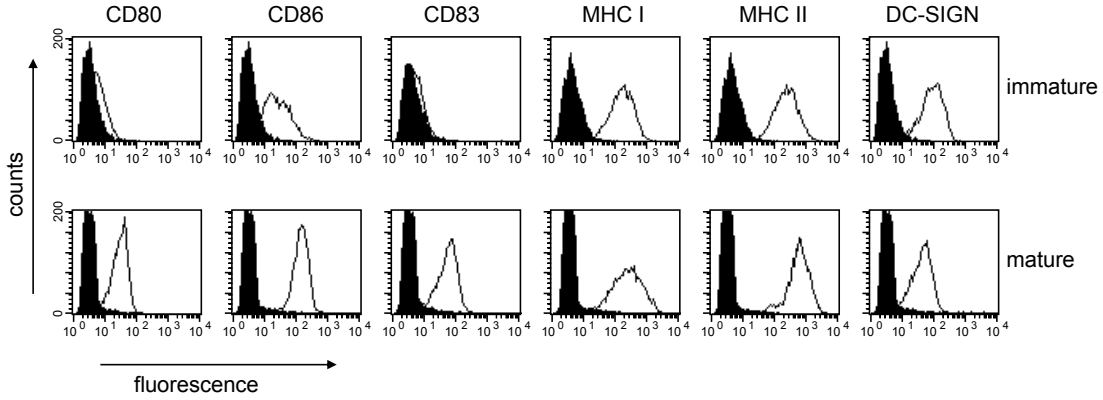

Figure 1. Characterization of immature and mature DCs. DCs were stained with monoclonal antibodies against antigen presentation molecules, costimulatory molecules, and DC-specific molecules, and measured by flow cytometry. Filled histograms represent isotype controls.

Adhesion and migration of DCs on fibronectin

We compared the adhesive and migratory capacity of immature versus mature DCs on fibronectin. When plated on fibronectin, immature DCs were subject to immediate and elaborate cell spreading, whereas mature DCs retained their veiled phenotype. Immature DCs seeded on fibronectin for 1 h began to form podosome-like structures recognizable as a dense core of actin surrounded by a ring of vinculin (Fig. 2A and B). These adhesive structures were not present in DCs with a "mature" phenotype (Fig. 2C and D). Prolonged incubation on fibronectin (2 h) resulted in more polarized immature DCs expressing podosomes, whereas mature DCs maintained their veiled phenotype and showed no podosome structures (Fig. 2E-H).

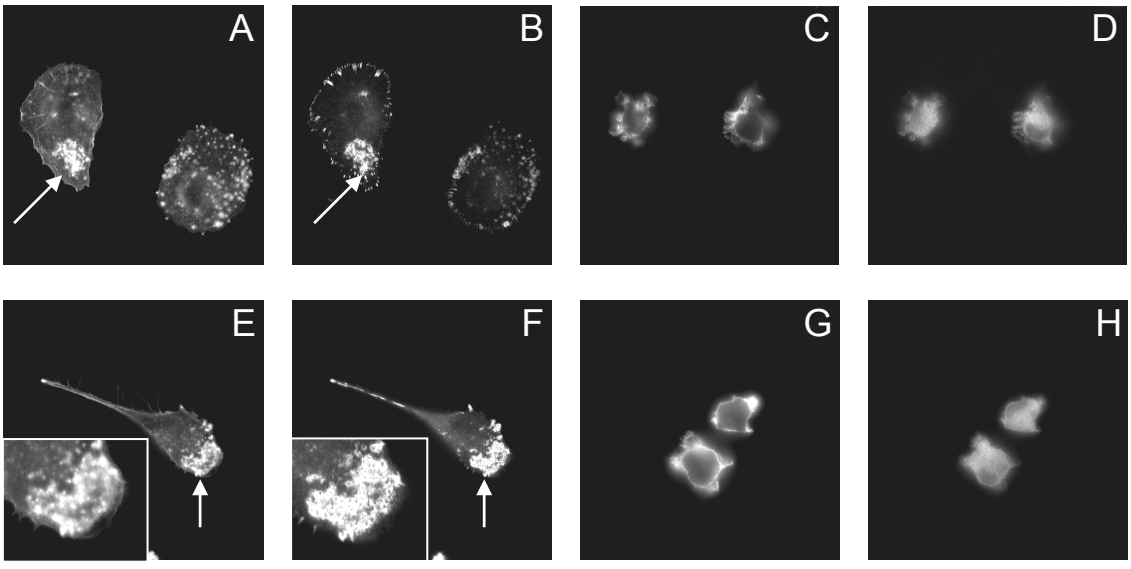

Figure 2. Morphology of DCs on fibronectin. Immature DCs (A, B, E, and F) and mature DCs (C, D, G, and H) were plated on fibronectin-coated coverslips for 1 (A-D) and 2 h (E-H), and subsequently stained with phalloidin (stains actin; A, C, E, and G) and anti-vinculin antibody (B, D, F, and H). Arrows indicate the podosomes. Inserts represent enlargements of the podosome structures.

Likewise, a larger proportion of immature DCs (80%, Fig. 3A) than of mature DCs (20%, Fig. 3B) bound to this ECM molecule. Consistent with the high adhesive properties of immature DCs, little or no migration was observed in this population (Fig. 3C). In contrast, mature DCs were highly motile and showed random migration at a mean speed of 5 μm/min (Fig. 3D and data not shown).

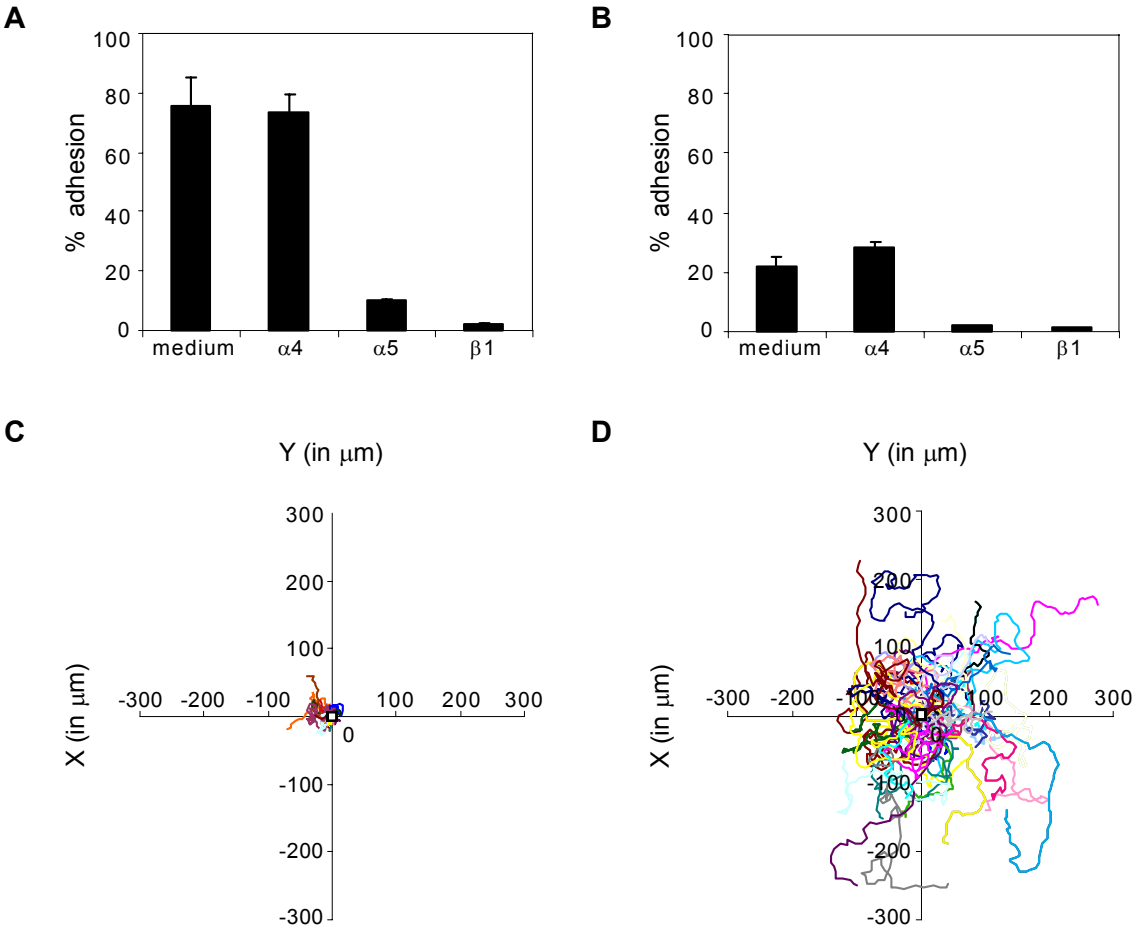

Figure 3. Adhesion and migration of DCs to fibronectin. Adhesion in the absence or presence of blocking antibodies against the α4, α5, and ß1 integrin chains (A, immature DCs; B, mature DCs). Results are expressed as the mean percentage of adhesion of triplicate wells. Data are representatives of three experiments. Migration of DCs on fibronectin (C and D). Plot of 50 individual immature (C) and mature (D) DC tracks (recording time 60 min), which are aligned at their starting positions.

Adhesion of mature DCs to fibronectin is enhanced by a β1-activating antibody

Adhesion of mature DCs was clearly reduced compared to immature DCs although both DC populations expressed comparable levels of β1. To exclude the possibility that β1 on mature DCs was somehow deficient in mediating adhesion, we investigated whether the β1-activating antibody TS2/16 could promote adhesion of mature DCs. Indeed adhesion of mature DCs to fibronectin was markedly enhanced by TS2/16. At the same time the adhesive capacity of immature DCs was not further induced, indicating that binding of immature DCs

to fibronectin was already maximal in medium conditions (Fig. 4). The TS2/16-induced adhesion of mature DCs was completely α5β1-mediated, since α5-blocking antibodies effectively blocked this interaction. These data indicate that α5β1 expressed on mature DCs is not defective in mediating adhesion. While cell-substrate adhesion not solely depends on integrin expression, but also on integrin distribution, and the activation state of the integrin (Huttenlocher *et al.*, 1996; Palecek *et al.*, 1997; van Kooyk & Figdor, 2000), these data suggest that β1 becomes either differentially distributed or less active upon DC maturation.

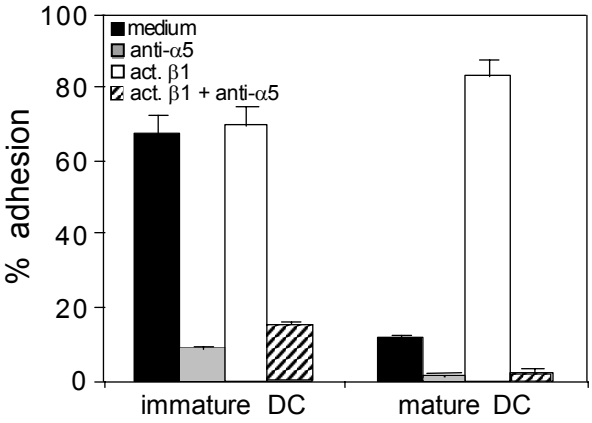

Figure 4. Adhesion of DCs after stimulation of the ß1 integrins. DC adhesion in the absence or presence of ß1-activating and α5-blocking antibodies. Results are expressed as the mean percentage of adhesion of triplicate wells. Data are representatives of three experiments.

The activation state of β1 is reduced upon DC maturation

To determine whether the reduced adhesion of mature DCs is due to decreased activation of β1, both immature and mature DCs were stained with the β1 antibodies AIIB2 (total β1) and 12G10. It has previously been shown that the 12G10 antibody recognizes a unique cation-regulated epitope on the β1 A-domain. The induction of this epitope correlates with the active conformation of the integrin (Mould *et al.*, 1995). Flow cytometric analysis revealed comparable levels of total β1 between immature and mature DCs. In contrast, a decrease in expression of the activation epitope was observed on mature DCs relative to immature DCs. These differences in β1 activity on immature versus mature DCs most likely account for their distinct adhesive and migratory potency on fibronectin and demonstrate that the regulation of β1 integrin activity is a key determinant of DC behavior.

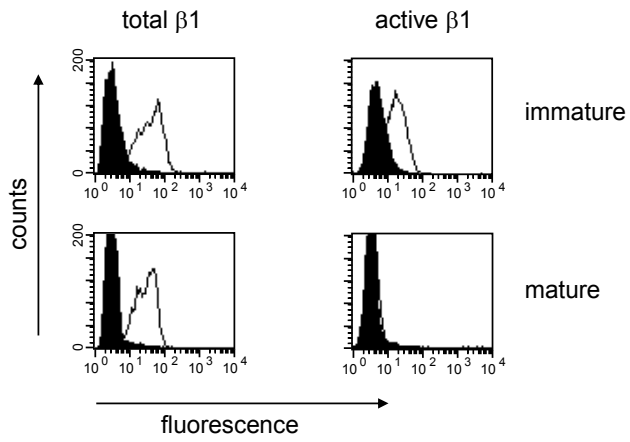

Figure 5. Expression of the activation epitope 12G10 on DCs. DCs were stained with the monoclonal antibodies against total β1 and the active conformation of the β1 A-domain and measured by flow cytometry. Representative expressions (1 out of 6) are shown. Filled histograms represent isotype controls.

Active β1 integrins localize to podosomes

Since immature DCs express active β1 integrins and form podosomes we investigated whether active β1 localizes preferentially to these adhesion structures. Therefore immature DCs were seeded on fibronectin-coated surfaces, incubated for 2 h, and stained for actin, total β1, and active β1. Immature DCs formed actin-rich dots corresponding to podosome structures (Fig. 6A and C). Cell surface localization of total β1 exhibited a uniformly expression pattern (Fig. 6B). No enrichment of total β1 was observed in podosomes. Staining for active β1 by the 12G10 antibody showed that it was primarily localized around actin cores in podosomes (Fig. 6D). These findings indicate that adhesion of immature DCs to fibronectin is established via active β1 integrins present in podosome structures.

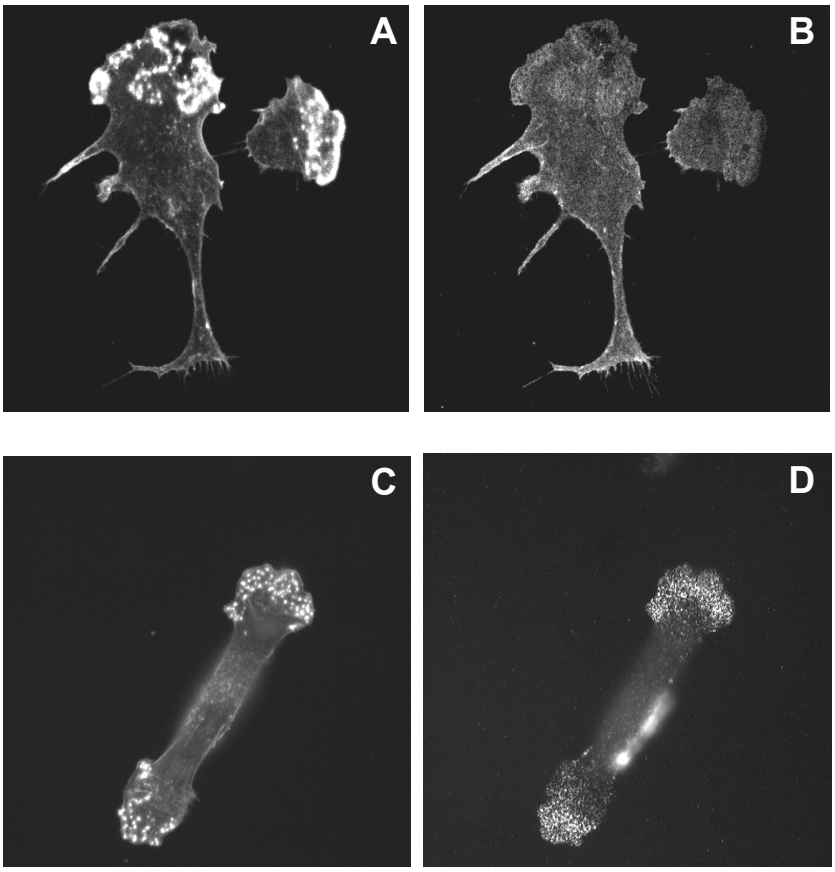

Figure 6. Localization of the activation epitope 12G10 on DCs. DCs were seeded on fibronectin-coated coverslips for 2 h and double stained for actin (A) and total β1 (B), and for actin (C) and the active conformation of the β1 A-domain (D).

DISCUSSION

The adhesion of immature DCs to fibronectin is considerably different from their mature counterparts; immature DCs adhere to fibronectin, whereas the adhesion of mature DCs is remarkably reduced. Here we have investigated to what extend either the expression level or the activation state of the β1 integrin contributes to these different adhesive capacities.

The expression level of the integrin β1 chain was comparable between immature and mature DCs, indicating that other factors than integrin expression modulate the difference in adhesion. Next to integrin expression levels, integrin activity and distribution can also be important modulators of adhesion (Huttenlocher *et al.*, 1996; Palecek *et al.*, 1997; van Kooyk & Figdor, 2000). For example, activation of αLβ2 results in a conformational change of the molecule and the expression of a Ca^{2+}-dependent and a Mn^{2+}-dependent epitope. Activation of αLβ2 and subsequent ligand binding result from conformational changes that increase both the affinity, but also the lateral multimerization of αLβ2 (avidity), and modulate adhesion (Binnerts & van Kooyk, 1999). Changes in integrin affinity and avidity vary with integrin type and the cellular context. While affinity and avidity changes play the most prominent role in the regulation of β2 integrins, the involvement of these changes in adhesion have also been shown for β1 integrins (Lobb *et al.*, 1995; Stewart & Hogg, 1996). The integrin α4β1 can acquire multiple activation states with different affinities (Jakubowski *et al.*, 1995). Likewise, α5β1 has at least two conformations, distinguished by the antibody 12G10, which binds to a cation-regulated epitope on the β1 A-domain (Mould *et al.*, 1995). High binding of 12G10 represents an "active" conformation, whereas an "inactive" state is recognized by low binding of 12G10 (Mould *et al.*, 2002). By staining immature and mature DCs with this 12G10 antibody, we showed that mature DCs expressed lower levels of active β1 than immature DCs. Consistent with this notion, we showed in adhesion assays that the β1-activating antibody TS2/16 could enhance the adhesion of mature DCs up to levels of immature DCs. These findings imply that the activation state of β1, rather than expression per se, is regulated upon DC maturation and modulates DC adhesion and migration.

Several studies have shown that cell surface distribution of integrins is also essential for their adhesive function. As example, reorganization of the spatial distribution of αLβ2 by a Rap1-binding molecule regulates lymphocyte adhesion (Katagiri *et al.*, 2003). Clustering of αLβ2 on the cell surface not only strengthens cell-cell adhesion but is also essential for αLβ2 activation and ligand binding. Expression of the Ca^{2+}-dependent epitope of αLβ2 is associated with a clustered state of αLβ2, which is a prerequisite for ligand binding (van Kooyk *et al.*, 1994; Binnerts & van Kooyk, 1999). Furthermore the distribution of αLβ2 is regulated upon DC differentiation (A. Cambi, personal communication). αLβ2 distribution changes from a clustered state in monocytes to a random distribution in immature DCs. The regulation of β1 activity during DC maturation might be induced by a difference in cell surface distribution.

Adhesion of immature DCs to fibronectin results in the assembly (1 h) or presence (2 h) of podosomes, which were absent on DCs with a mature phenotype. These findings extended the study of Burns et al., who related podosome formation to cells with an immature phenotype (Burns *et al.*, 2001). Podosomes consist of a core of actin filaments and actin associated proteins surrounded by a ring of vinculin and talin, and interact with the substratum via integrins (Marchisio *et al.*, 1988; Linder & Aepfelbacher, 2003). We showed by fluorescence microscopy an enrichment of active β1 in these podosome structures, indicating that the activity as well as the distribution of β1 account for the opposite adhesive capacities of immature and mature DCs. Because transmission electron microscopy is a more sensitive technique to measure distribution patterns of cell surface molecules, we will use this technique to investigate whether β1 is differently distributed in immature and mature DCs.

Burns and colleagues have suggested that podosome structures play a role during the migratory phase of DCs (Burns *et al.*, 2001). However, here we show that mature DCs, which lack podosomes, are highly migratory, whereas immature DCs containing this structure are strongly adherent. Since mature DCs use transient interactions with fibronectin to migrate, we speculate that podosomes regulate strong adhesion still allowing amoeboid crawling of cells (speed 0.5 μm/min, De Vries *et al.*, 2003), but not the high speed migration seen in mature DCs (5 μm/min, De Vries *et al.*, 2003).
Activation of integrins and subsequent signal transduction is regulated by membrane recruitment of cytoplasmic proteins. One of such proteins is Cytohesin-1, which binds to αLβ2 and increases its avidity (Kolanus *et al.*, 1996; Geiger *et al.*, 2000; Weber *et al.*, 2001). Another recently described protein is CYTIP, which is expressed in hematopoietic cells and only associates with the plasma membrane in cells that adhere to integrin ligands (Boehm *et al.*, 2003). CYTIP interacts with cytohesin-1 and detachment of this complex from the plasma membrane abrogates β2 integrin adhesion, but has no effect on β1-dependent adhesion. The regulated expression of these molecules in DCs suggests a dynamic control of β2-integrin-mediated adhesion. It would be interesting to investigate whether a comparable mechanism exists for β1 integrins. Cytoplasmic proteins that can bind the tail of β1 integrins and modulate their function are talin and paxillin (Schaller *et al.*, 1995; Rock *et al.*, 1997). Upon DC maturation, the actin-bundling protein fascin and the microfilament-associated protein palladin are upregulated (Mosialos *et al.*, 1996; Mykkanen *et al.*, 2001). Future investigations will demonstrate whether one of these proteins modulate integrin-mediated DC adhesion and migration. Recently, Cook et al. showed that the tetraspanin molecule CD9 associates with α5β1 when transfected in CHO cells (Cook *et al.*, 2002). Though no evidence is yet available on the effect of tetraspanins on integrin conformation, Cook and colleagues demonstrated that CD9 association modulates adhesion to fibronectin. We observed no colocalization of CD9 with α5β1 (data not shown) indicating that this tetraspanin molecule does not appear to be involved in immature and mature DC adhesion to fibronectin.
In this report we demonstrate that the reduced adhesion of mature DCs versus immature DCs is predominantly determined by the activation state of the β1 integrin rather than by β1 integrin expression levels. In addition we show that immature DCs, seeded on fibronectin exhibit podosomes that contain active β1 integrins. The migration of DCs upon maturation coincides with inactivation of the β1 integrin. Future experiments will be aimed at understanding the molecular mechanism responsible for this remarkable change in activation of the β1 integrin.

ACKNOWLEDGMENTS
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Chapter 5

DC-SIGN-ICAM-2 interaction mediates dendritic cell trafficking

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ABSTRACT

Dendritic cells (DCs) are recruited from blood into tissues to patrol for foreign antigens. After antigen uptake and processing DCs migrate to the secondary lymphoid organs to initiate immune responses. We now show that DC-SIGN, a DC-specific C-type lectin, supports tethering and rolling of DC-SIGN-positive cells on the vascular ligand ICAM-2 under shear flow, a prerequisite for emigration from blood. The DC-SIGN/ICAM-2 interaction regulates chemokine-induced transmigration of DCs across both resting and activated endothelium. Thus, DC-SIGN is central to the unusual trafficking capacity of DCs, further supported by the expression of DC-SIGN on precursors in blood, and on immature as well as mature DCs in both peripheral and lymphoid tissues.

INTRODUCTION

A fundamental aspect of dendritic cell (DC) function to control immunity is their capacity to migrate (Banchereau & Steinman, 1998). Immature DCs migrate from the blood into peripheral tissues to exert a continuous surveillance for incoming foreign antigens. This migration from blood is tightly regulated to provide a rapid response to inflammatory signals. Immature DCs in peripheral tissues are highly proficient in antigen capture and processing (Inaba *et al.*, 1993; Svensson *et al.*, 1997). Upon receiving an activation signal, immature DCs mature and specifically migrate from the periphery to secondary lymphoid organs for interaction with specific T cells and initiation of an immune response (Banchereau & Steinman, 1998). Little is known about the molecular basis of DC migration and whether DCs express specific molecules that regulate their adhesion and migratory capacity. We have recently identified the human DC-specific adhesion receptor DC-SIGN, a C-type lectin, through its high affinity interaction with ICAM-3 and which enables transient DC-T cell interactions, thus facilitating primary immune responses (Geijtenbeek *et al.*, 2000a). Furthermore, DC-SIGN functions as a novel HIV-1 trans-receptor important in the dissemination of HIV-1 (Geijtenbeek *et al.*, 2000b). HIV-1 is captured on DCs present in the periphery by DC-SIGN and thus transported by DCs that migrate into lymphoid tissues where DC-SIGN-associated HIV-1 efficiently infects target $CD4^+$ T cells.
Immature DCs express high levels of DC-SIGN (Geijtenbeek *et al.*, 2000b), and since several lectins have also been reported to function as rolling receptors allowing leukocyte transendothelial migration (Vestweber & Blanks, 1999), we hypothesized that DC-SIGN might play a role in the specific migratory capacity of DCs. We investigated whether ICAM-2 functions as a ligand for DC-SIGN, since ICAM-2 has a high homology with ICAM-3 (de Fougerolles *et al.*, 1993) and, in contrast to ICAM-3, is abundantly expressed by vascular as well as lymphoid endothelium (Nortamo *et al.*, 1991). Strikingly, DC-SIGN functions not only as the primary receptor for ICAM-2 on DCs, but also mediates DC rolling and transendothelial migration, thus demonstrating that DC-SIGN is crucial in the specific migratory processes of DCs.

MATERIALS AND METHODS

Adhesion

Fluorescent bead adhesion assays were performed as described (Geijtenbeek *et al.*, 1999; Geijtenbeek *et al.*, 2000a). Briefly, carboxylate-modified Transfluospheres (488/645nm; Molecular Probes, Eugene, OR) were coated with ICAM-2-Fc. 50,000 cells were preincubated with mAb at 20 μg/ml for 10 min at room temperature. ICAM-2-Fc-coated fluorescent beads (20 beads/cell) were added, and the suspension was incubated for 30 min at 37ºC. Adhesion was determined by measuring the percentage of cells which had bound fluorescent beads by flow cytometry using the FACScan (Becton Dickinson, Oxnard, CA). Ca^{2+} affinity was determined by measuring the binding of DCs to ICAM-coated beads at different Ca^{2+} concentrations. Specificity is determined in the presence of antibodies to DC-SIGN (AZN-D1; 20 μg/ml). The resulting curves for both ICAM-2 and ICAM-3 were fitted to the equation for second order dependence to Ca^{2+} (fractional binding)$=[Ca^{2+}]^2/((K_{Ca})^2+[Ca^{2+}]^2)$ (Mullin *et al.*, 1997). Ligand affinity was determined by titrations of soluble ICAM-1, -2, -3-Fc and measuring the binding to either LFA-1- or DC-SIGN-transfectants. Binding was measured after 30 min at 37ºC by staining the ligand Fc chimeras with FITC-conjugated goat-anti-human Fc antibodies and determining the fraction

of labeled cells by flow cytometry. Specificity was determined by measuring in the presence of blocking antibodies to either LFA-1 (NKI-L15; 20 μg/ml) or DC-SIGN (AZN-D1; 20 μg/ml).

Laminar flow assay

The laminar flow assay was performed as previously described (Alon *et al.*, 1995; Chen *et al.*, 1999). Briefly, a polystyrene plate was coated with either ICAM-1-, ICAM-2-, or ICAM-3-Fc bound to immobilized protein A. The plate was assembled in a parallel-plate laminar flow chamber (260-μm gap) and mounted on the stage of an inverted phase contrast microscope (Diaphot 300; Nikon, Tokyo, Japan). K562-DC-SIGN transfectants (100 to 200 cells per field of view) were introduced into the flow chamber for 1 min at a constant low shear flow (0.25 dyn/cm^2) before being subjected to an incremental increase in shear stress. Shear flow was generated and controlled with an automated syringe pump (Harvard Apparatus, Natick, MA) attached to the outlet side of the flow chamber. The wall shear stress was increased step-wise every 5 s until it reached 10 dyn/cm^2. At the end of each 5-s interval at a particular shear stress, the number of cells that remained bound (stationary or rolling) was determined relative to the number of cells that had accumulated at low shear flow on the ICAM-coated field. Cellular interactions were determined on two representative fields of view (each typically 0.34 mm^2 of area). Displacement velocities were determined only for persistently-rolling cells which remained adherent throughout the entire shear stress assay.

Adhesion and transendothelial migration

Heterotypic cell clustering of DCs with the endothelial HMEC-1 cell-line was performed as described previously for DC-T cell clustering (Geijtenbeek *et al.*, 2000a). Briefly, HMEC-1 cells, cultured in MCDB131 medium (supplemented with 10% FCS), were labeled with the fluorescent dye hydroethidine. DCs were labeled with the fluorescent dye sulfofluorescein. The cells were incubated with or without blocking antibodies (20 μg/ml) at 37ºC. Heterotypic cell clustering was measured by flow cytometry. Transwell 24 well plate (8 μm pore) were coated with fibronectin (20 μg/ml; 1 h at 37ºC). 40,000 HMEC-1 cells were seeded on the inserts and after 24 h the cells were activated with TNF-α (5 ng/ml) for 16 h. 200,000 DCs were added to the monolayer of endothelial HMEC-1 cells. The lower chamber contained 10 ng/ml SDF-1. After 20 h at 37ºC, the number of transmigrated DCs (lower chamber) was determined by flow cytometry. The transendothelial migration was measured in the presence of blocking antibodies (40 μg/ml).

Isolation of DC-SIGN-positive cells from blood

PBMC obtained from a buffy coat were depleted for CD3-, CD20- and CD56-positive cells with magnetic beads (Dynal, Oslo, Norway) according to standard procedures. The depleted cell fraction was incubated with FITC-conjugated anti-DC-SIGN Ab (AZN-D1) and the DC-SIGN-positive cells were sorted by flow cytometry with the Coulter Epics Elite (Coulter, Hialeah, FL). The sorted cell fraction was analyzed for the expression of DC-SIGN (AZN-D1), CD14 and CD83 (Immunotech, Fullertone, CA), CD80 (Becton Dickinson, Oxnard, CA), CD86 (BD Pharmingen, San Diego, CA), CD83 (Immunotech, Marseille, France) MHC class I (W6/32), MHC class II (Q5/13), CD11b (KIM225), and CD11c (SHCL3) by three-color fluorescence staining using FITC-, PE- and Cy5PE-conjugated directly labeled antibodies.

Immunohistochemical staining

Cryosections (8 μm) of tissues were fixed in 100% aceton for 10 min, washed with PBS and incubated with the primary antibody 10 μg/ml for 60 min at 37ºC. After washing, the final staining was performed with the ABC-PO/ABC-AP Vectastain kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol. Nuclear counterstaining was performed using hematoxylin.

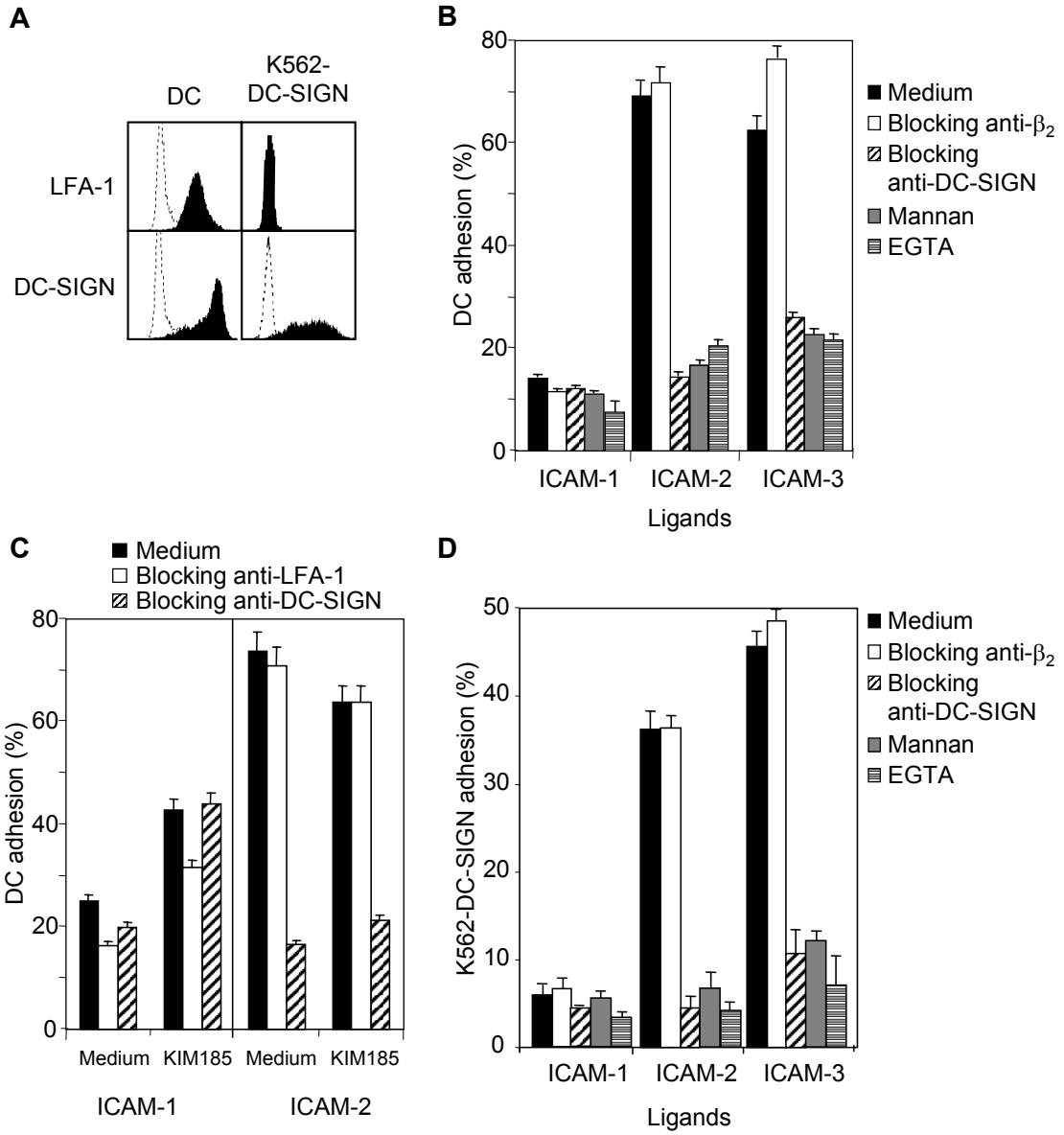
RESULTS

ICAM-2, a novel counterstructure for DC-SIGN

Monocyte-derived immature DCs (Romani *et al.*, 1994; Sallusto & Lanzavecchia, 1994) express high levels of DC-SIGN and moderate levels of LFA-1, the primary ICAM-2 receptor on leukocytes (Fig. 1A). Immature DCs strongly bind ICAM-2 comparable to our results for ICAM-3 (Geijtenbeek *et al.*, 2000a) (Fig. 1B), as determined by the novel ICAM-Fc-coated fluorescent bead adhesion assay (Geijtenbeek *et al.*, 2000a). Adhesion of DCs to ICAM-2 is completely mediated by the DC-specific C-type lectin DC-SIGN and not by LFA-1 (Fig. 1B) since antibodies to DC-SIGN inhibit the adhesion in contrast to antibodies to the β2-integrin chain (Fig. 1B) or the LFA-1 α-chain (results not shown). The interaction with ICAM-2 is also inhibited by either EGTA or mannan (Fig. 1B), indicating that the adhesion is mediated by the C-type lectin domain of DC-SIGN. Even after activation of LFA-1 on DCs with the stimulatory anti-β2 antibody KIM185, adhesion to ICAM-2 remains completely DC-SIGN-specific (Fig. 1C).

In order to determine the relative affinity of DC-SIGN for ICAM-2 without any contribution from LFA-1, we generated a stable DC-SIGN-positive transfectant from the LFA-1-negative cell-line K562 (Fig. 1A). This DC-SIGN-positive transfectant strongly binds to both ICAM-2 and ICAM-3, but not to ICAM-1, and the binding is inhibited by either EGTA or mannan as was observed for DCs (Fig. 1D). The relative binding affinity of DC-SIGN for soluble ICAM-2 (IC50 of 1 μg/ml) is higher than that of LFA-1 on the HSB T cell-line (IC50 of 8 μg/ml) (Fig. 2A and B) demonstrating that DC-SIGN is the primary receptor for ICAM-2. Until now the primary receptor for ICAM-2 has been thought to be the β2 integrin LFA-1. Moreover the affinity of DC-SIGN for ICAM-2 (IC50 of 1 μg/ml) is higher than that for ICAM-3 (IC50 of 6 μg/ml) (Fig. 2A). Similar to the binding of ICAM-3, the ICAM-2 interaction with DC-SIGN requires two Ca^{2+}-ions as demonstrated by the second order Ca^{2+} ion-dependency (Fig. 2C) that is characteristic for C-type lectins (Drickamer, 1995; Geijtenbeek *et al.*, 2000a). Thus, we have identified ICAM-2 as a novel endothelial counter structure for DC-SIGN.

Figure 1. ICAM-2 is a novel endothelial counter structure for the DC-specific C-type lectin DC-SIGN. (A) Immature DCs express high levels of DC-SIGN (AZN-D1) and intermediate levels of LFA-1 (NKI-L7). K562-DC-SIGN transfectants express high levels of DC-SIGN but no LFA-1. Transfectants were generated as previously described (Geijtenbeek *et al.*, 2000b). Dotted line represents isotype control and filled histogram indicates specific antibody staining of gated population. (B) Immature DCs bind both ICAM-2 and ICAM-3 through DC-SIGN. Cells were allowed to bind ICAM-Fc-coated fluorescent beads for 30 min at 37°C. Adhesion was measured by flow cytometry. The adhesion was determined in the presence of either mannan (5 mM), EGTA (5 mM) or blocking antibodies to DC-SIGN (AZN-D1, 20 μg/ml) or β2 integrins (AZN-L19, 20 μg/ml). One representative experiment out of three is shown. (C) DC-SIGN is the major ICAM-2 receptor on immature DCs even after LFA-1 activation. LFA-1 on DCs was activated by the stimulatory anti-β2 Ab KIM185 and adhesion to both ICAM-1 and ICAM-2 was determined in the presence of either anti-DC-SIGN (AZN-D1) or anti-LFA-1 (NKI-L15) Ab, as described in Fig. 1B. One representative experiment out of two is shown. (D) DC-SIGN expressed by K562 transfectants bind to ICAM-2 as well as to ICAM-3. Adhesion was determined as described in Fig. 1B. One representative experiment out of three is shown.



DC-SIGN mediates rolling on ICAM-2

A prerequisite for vascular migration of circulating immune cells from blood into tissues is establishment of shear-resistant contacts with blood vessel walls for transmigration through their endothelial lining (Springer, 1994). We investigated whether the DC-SIGN/ICAM-2 interaction enables DCs to establish these shear-resistant contacts under physiological flow conditions by using the laminar flow chamber system, since ICAM-2 is abundantly expressed by vascular endothelium. This system has been extensively used to investigate leukocyte rolling mediated by receptors such as the selectins (Lawrence & Springer, 1991; Alon *et al.*, 1997; Smith *et al.*, 1999). We analyzed the adhesive properties of DC-SIGN-expressing K562 transfectants under shear flow to surfaces coated with similar densities of either the vascular endothelial ligands ICAM-1 or ICAM-2, or the resting T cell ligand ICAM-3. No contribution of the β2 integrin ICAM receptors, such as LFA-1 or Mac-1 is possible since K562 cells do not

endogenously express β2 integrins. DC-SIGN interacts only with ICAM-2 under shear flow (Fig. 3A), although DC-SIGN supports adhesion to both ICAM-2 and ICAM-3 equally under static conditions (Fig. 1D). Both the frequency of cell tethers as well as their resistance to detachment by incrementally increasing shear forces was augmented on ICAM-2 in contrast to ICAM-3 (Fig. 3A). Tethering and rolling on ICAM-2 is DC-SIGN-specific, since antibodies to DC-SIGN inhibited the observed interactions (data not shown). DC-SIGN binding to ICAM-2, but not to ICAM-3, supported continuous rolling of DC-SIGN-expressing transfectants, reminiscent of selectin-mediated leukocyte rolling (Fig. 3B and C). Average rolling velocity of DC-SIGN-positive cells at 1.5 and 2.0 dyn/cm^2 is 17.9±1.4 and 19.8±1.4 μm/s, respectively. Furthermore, DC-SIGN-expressing cells are recruited to the ICAM-2-coated surface, since the cells bound to ICAM-2 when introduced into the flow chamber under shear flow. ICAM-2 serves as a rolling ligand only for DC-SIGN and not for LFA-1, as the binding of LFA-1-expressing cells to ICAM-2 is abolished when shear stress was applied (data not shown), demonstrating that the DC-SIGN/ICAM-2 rolling interaction is a characteristic of the C-type lectin DC-SIGN and not of the integrin LFA-1. Thus, DC-SIGN enables DCs to interact with vascular endothelium under shear flow, a prerequisite for extravasation from blood into tissues.

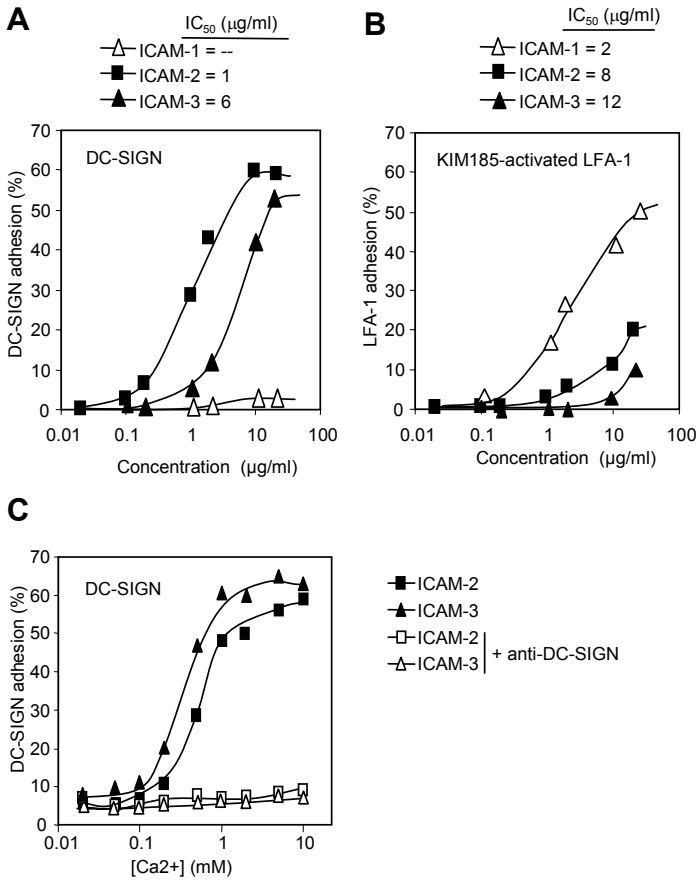


Figure 2. DC-SIGN has a higher affinity for both ICAM-2 and ICAM-3 than LFA-1. (A) DC-SIGN has the highest affinity for ICAM-2, intermediate for ICAM-3 and does not bind ICAM-1. Adhesion of soluble ICAM-2-Fc and ICAM-3-Fc to K562-DC-SIGN transfectants was measured. Binding was analyzed by flow cytometry after staining with FITC-conjugated anti-human Fc antibody. The expression level of DC-SIGN on K562 cells is similar to the level of LFA-1 on HSB cells (data not shown). One representative experiment out of three is shown (SD<5%). (B) LFA-1 has a low affinity for both ICAM-2 and ICAM-3 and high affinity for ICAM-1. LFA-1 on HSB cells was activated by the stimulatory anti-β2 integrin KIM185 antibody (10 μg/ml). The binding assay was performed similar as in Fig. 2A. One representative experiment out of three is shown (SD<5%). (C) DC-SIGN-mediated binding to both ICAM-2 and ICAM-3 is Ca^{2+}-dependent and DC-SIGN has a slightly higher KCa for ICAM-2 (0.28 mM) than for ICAM-3 (0.22 mM). Adhesion was determined by the fluorescent bead adhesion assay at different Ca2+ concentrations. One representative experiment out of three is shown.

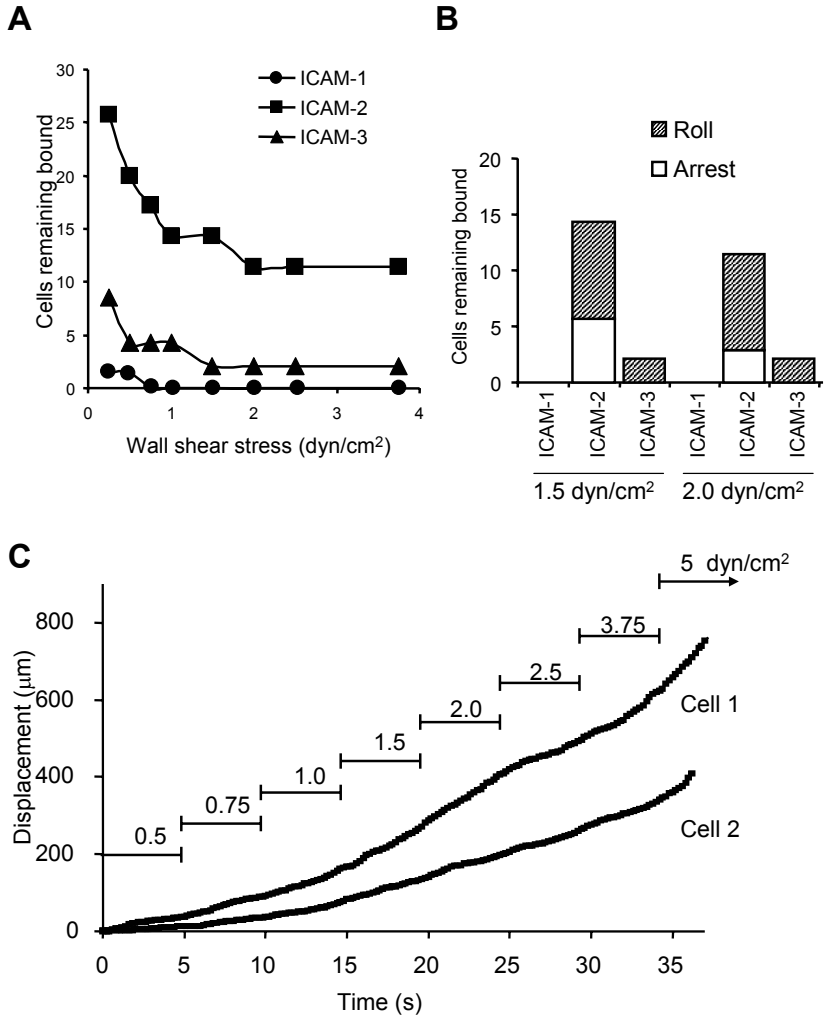


Figure 3. DC-SIGN mediates tethering to and rolling on ICAM-2 but not on ICAM-3 under physiological shear flow. (A) DC-SIGN mediates tethering to ICAM-2 but not to ICAM-3. All adhesions were confirmed to be DC-SIGN-specific by blocking with the anti-DC-SIGN antibody AZN-D1. DC-SIGN-mediated tethering to ICAM-2 was 5% when the shear flow was increased to 10 dyn/cm^2 (data not shown). One representative experiment out of three is shown. (B) DC-SIGN mediates rolling on ICAM-2 coated surfaces. Fractions of adherent K562-DC-SIGN cells that maintained steady rolling or were arrested on the ICAM substrate at the indicated shear stresses are depicted. The fractions were determined in two representative fields at shear stresses of 1.5 and 2.0 dyn/cm^2. One representative experiment out of three is shown. (C) Displacement with time of individual DC-SIGN-transfected cells rolling on ICAM-2 and subjected to incremental shear stresses at the indicated time points. One representative experiment out of two is shown.

DC-SIGN mediates transendothelial migration of DCs

We next investigated whether DC-SIGN is necessary for the endothelial transmigration of DCs. Both resting and Tumor Necrosis Factor (TNF)-α–activated endothelial cells express intermediate levels of ICAM-1 and high levels of ICAM-2 but no ICAM-3, whereas TNFα-activation strongly upregulates both ICAM-1 and VCAM-1 expression (Fig. 4A). DC bind to resting endothelial cells through DC-SIGN, whereas binding to TNFα-activated endothelium is both DC-SIGN- and β1/β2 integrin-dependent (Fig. 4B) due to upregulation of the β1 and β2 integrin ligands VCAM-1 and ICAM-1, respectively.

Subsequently we investigated the involvement of DC-SIGN in the endothelial transmigration of DCs. Members of the chemokine family, such as SDF-1, have been shown to direct specific transendothelial migration of DCs (Sallusto & Lanzavecchia, 1994; Banchereau & Steinman, 1998; D'Amico *et al.*, 1998; Delgado *et al.*, 1998). In a physiological system, SDF-1 induced migration of DCs across both resting and TNFα-activated endothelium (Fig. 4C). DC-SIGN also mediated transmigration to other chemokines such as MIP-1α and Rantes (data not shown). Both spontaneous and SDF-1-induced migration across resting endothelium was predominantly mediated by DC-SIGN, whereas the contribution of the β1/β2 integrins was minimal (Fig. 4C). SDF-1-induced migration of DCs across TNFα-activated endothelium was strongly dependent on DC-SIGN (Fig. 4C), despite the elevated levels of integrin ligands on the TNFα-activated endothelium (Fig. 4A). The discrepancy between the relatively minor contribution of DC-SIGN to the static adhesion of DCs to endothelium, compared to the major contribution of DC-SIGN to the transendothelial migration of DCs (Fig. 4B and C) demonstrates that the transient, more dynamic interactions, provided by DC-SIGN, are essential for transendothelial migration.

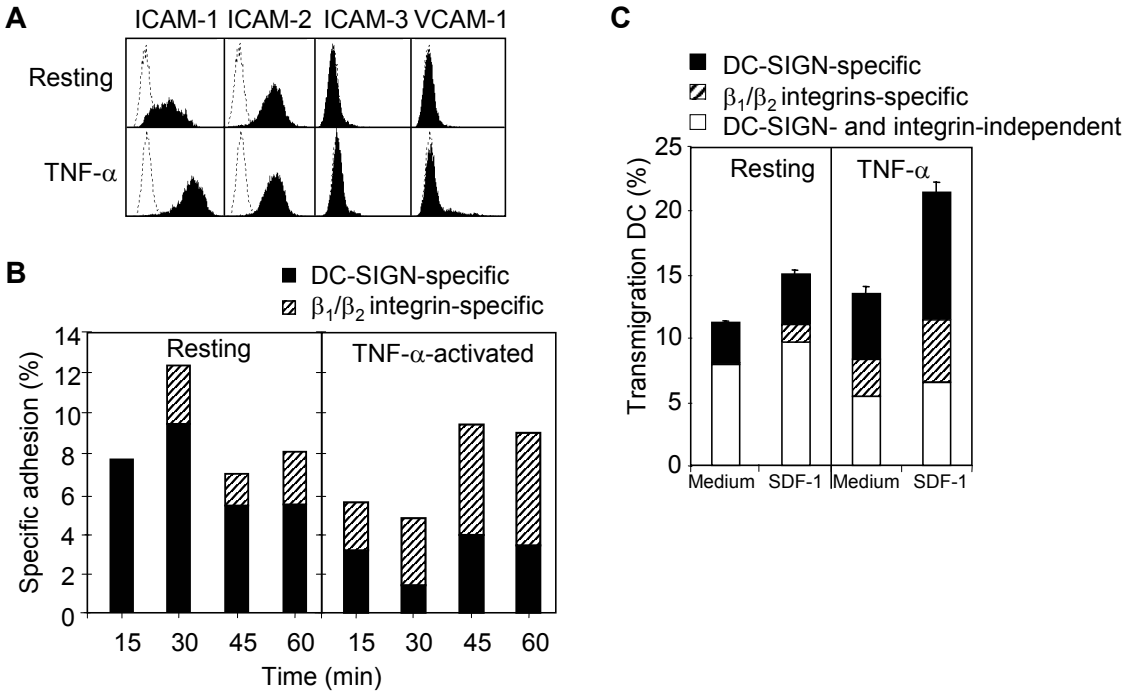


Figure 4. DC-SIGN mediates SDF-1 induced transmigration of DCs across both resting and TNFα-activated endothelium. (A) Expression levels of ICAM-1 (Rek-1), ICAM-2 (CBR-IC2/2) and VCAM-1 (4B9) on both resting and TNFα-activated endothelial HMEC-1 cells. (B) Adhesion of DCs to both resting and TNFα-activated endothelium is mediated by DC-SIGN. Adhesion specificity was determined in the presence of blocking anti-DC-SIGN (AZN-D1; 20 μg/ml) or anti-β1/β2 integrin (AIIB2 and AZN-L19; 20 μg/ml) Ab at 37°C. One representative experiment out of 2 experiments is shown. (C) DC-SIGN mediates transmigration of DCs across both resting and TNFα-activated endothelium in response to a SDF-1 gradient. The transendothelial migration was measured in the presence of antibodies to DC-SIGN (AZN-D1 and AZN-D2; 40 μg/ml), VLA-4 integrin (HP2/1; 40 μg/ml) and β2 integrins (AZN-L19; 40 μg/ml). One representative experiment out of three is shown.

DC-SIGN-positive precursors are present in blood

We next assessed the *in vivo* relevance of these interactions by immunohistochemistry. ICAM-2 is abundantly expressed on the endothelial linings of both vascular and lymphatic vessels *in vivo* (Fig. 5A), suggesting that ICAM-2/DC-SIGN interactions might play a role in the migration of DC-SIGN-positive blood precursors into peripheral tissues as well as of immature and mature DCs into lymphoid tissues. Indeed we found that in blood two DC-SIGN-positive subpopulations, $CD14^+$ and $CD14^-$, were present (Fig. 5B). Both cell populations express high levels of MHC class I, MHC class II, CD11b and CD11c, intermediate levels of CD83 and CD86, and no CD80 (Fig. 5C). This together with the expression of DC-SIGN on immature DCs in the periphery (Geijtenbeek *et al.*, 2000a) (Fig. 5D), and on mature DCs in the lymphoid organs (Geijtenbeek *et al.*, 2000a) (Fig. 5E), indicate that DC-SIGN/ICAM-2 interactions may regulate DC migration from blood into peripheral tissues and subsequently into lymphoid tissues.

DISCUSSION

DC-SIGN, a DC-specific adhesion receptor, and a Type II transmembrane mannose binding C-type lectin, regulates primary immune responses by establishing transient DC-T cell interactions through binding of the leukocyte counter structure ICAM-3 (Geijtenbeek *et al.*, 2000a). We now show that this novel DC-specific adhesion molecule recognizes with high affinity the endothelial counter-structure ICAM-2, also a well-known ligand of the β2 integrin LFA-1. In contrast DC-SIGN does not bind ICAM-1, the major ligand of LFA-1. Thus, DC-SIGN is the primary DC-specific receptor for both ICAM-2 and ICAM-3, even though DCs express LFA-1. Although DC-SIGN binds to both ICAM-2 and ICAM-3 under static conditions, under physiological flow conditions, only the DC-SIGN/ICAM-2 interaction resists shear stresses, allowing DC-SIGN-positive cells to tether to and roll along ICAM-2-coated surfaces. ICAM-2 serves as a rolling counter receptor for DC-SIGN but not for LFA-1 (data not shown) indicating that only DC-SIGN but not LFA-1 functions as a rolling receptor. ICAM-2 is abundantly expressed on endothelial blood vessels (Nortamo *et al.*, 1991), which would enable DC-SIGN-positive DCs to tether to and roll along vascular endothelium, a prerequisite for transendothelial migration into the peripheral tissues. DC-SIGN-mediated rolling on ICAM-2 was observed over the entire range of physiological shear stresses reported to occur in post-capillary venules that are known to support leukocyte emigration (Heisig, 1968). Thus, DC-SIGN is a DC-specific rolling receptor and apart from the P-, E- and L-selectins (Vestweber & Blanks, 1999), the only other known adhesion molecules involved in general leukocyte tethering and rolling on vascular endothelium were the α4-integrins VLA-4 ($\alpha4\beta1$), and $\alpha4\beta7$ (Springer, 1994; Alon *et al.*, 1995; Berlin *et al.*, 1995). DC-SIGN contains a C-type lectin domain, similar to the selectins, indicating that this particular class of lectin domains is well-suited to mediate rolling. The interaction of DC-SIGN with ICAM-2 resisted shear stress whereas binding of DC-SIGN to ICAM-3 was abolished under similar conditions. DC-SIGN-mediated binding to ICAM-3 *in vivo* does not need to resist shear forces since ICAM-3 is absent on endothelial cells and the DC-SIGN/ICAM-3 interaction mediates DC-T cell clustering in secondary lymphoid tissues, which are not exposed to shear forces.

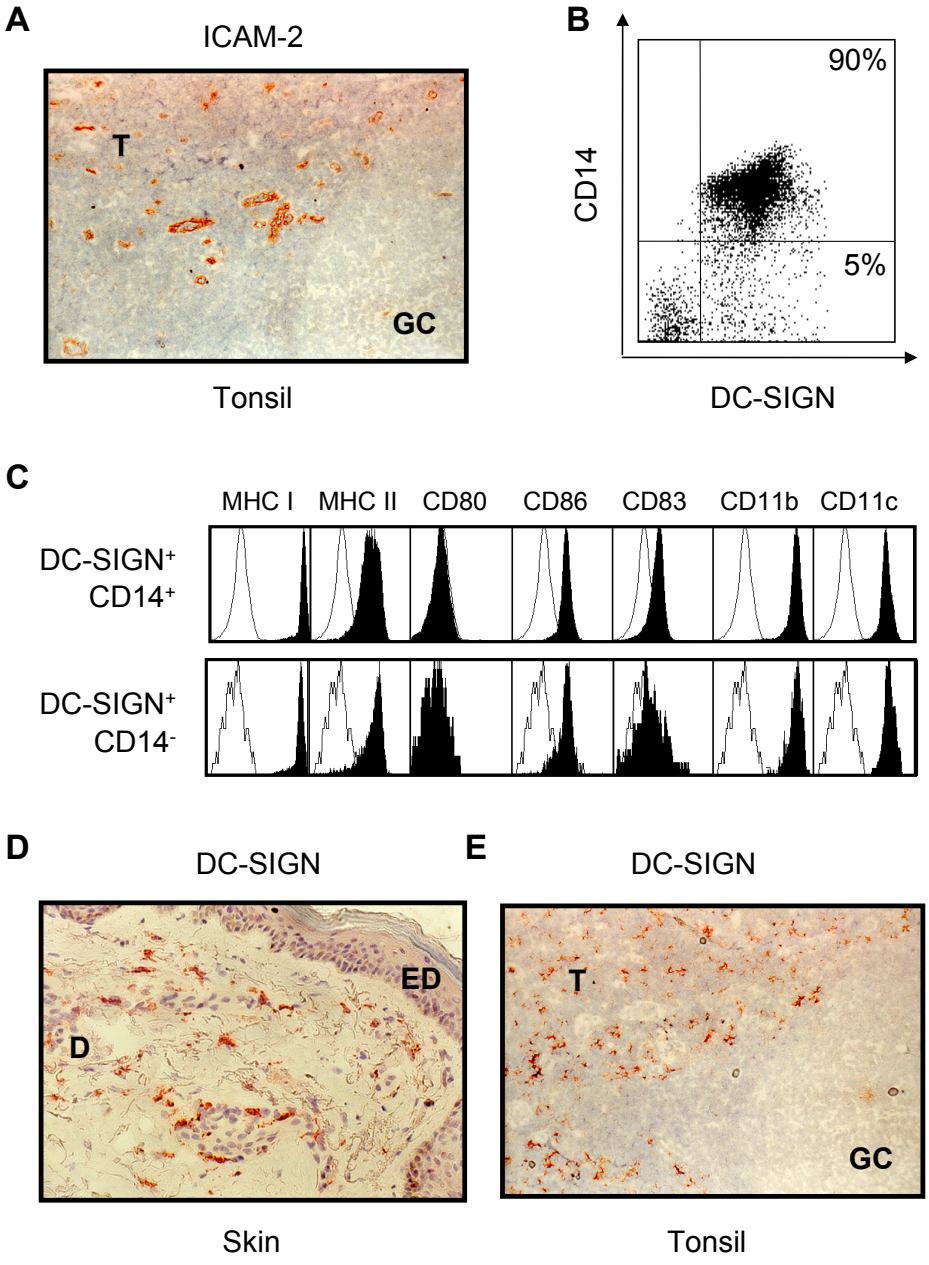


Figure 5. DC-SIGN is expressed by DCs precursors in blood, immature DCs in skin and mature DCs in lymphoid tissues. (A) ICAM-2 is abundantly expressed on endothelial vascular (HEV) cells as well as lymphatic vessels in tonsils. Tissue sections of tonsil were stained for the expression of ICAM-2 (CBR-IC2/2). (B) Blood contains two DC-SIGN-positive cell populations which are CD14-positive (90%) and –negative (5%), respectively (ratios between CD14-positive and CD14-negative varies between blood donors from 20:1 to 3:1). The DC-SIGN-positive fraction, representing 0.04% of total PBMC, was isolated from CD3/CD20/CD56-depleted PBMC by positive flow cytometric cell-sorting after staining with FITC-conjugated anti-DC-SIGN Ab (AZN-D2). One representative experiment out of three is shown. (C) DC-SIGN-positive blood precursors express both antigen-presenting and co-stimulatory molecules. DC-SIGN-positive blood cells (Fig. 5B) were stained for the expression of different markers. (D-E). DC-SIGN is abundantly expressed by immature dermal DCs in the skin (D=dermal, ED=epidermal) (D) and by mature DCs in tonsils (G=germinal center, T=T cell area) (E). Tissue sections of tonsil were stained for the expression of DC-SIGN (AZN-D1).

Monocytes have been demonstrated to be precursors of DCs (Randolph *et al.*, 1998a) and we have demonstrated that DC-SIGN is rapidly upregulated on monocytes in the presence of IL-4 and GM-CSF (Geijtenbeek *et al.*, 2000a). Thus, DC-SIGN-upregulation by cytokine mediators may induce migration of precursor DCs from blood into the periphery. The presence of DC-SIGN-positive cells in blood further supports our model that under physiological circumstances DC-SIGN/ICAM-2 interactions mediate rolling along endothelial linings and transmigration of DCs into the periphery. The ICAM-2/DC-SIGN-mediated tethering to and rolling interactions along the blood vessels enable these cells to react swiftly to inflammatory chemoattractants that direct extravasation of DCs into peripheral tissues as demonstrated by the abundant expression of DC-SIGN on DCs present in these tissues (Geijtenbeek *et al.*, 2000a) (Fig. 5D). Upon antigen challenge these immature DCs will mature and migrate to the lymphoid tissues (Fig. 5E) where ICAM-2 is abundantly expressed on the endothelial linings in both vascular and lymphatic vessels (Fig. 5A). Therefore these results demonstrate a central role for ICAM-2 in DC-specific migration from blood into periphery, inflamed or not inflamed, and subsequently of immature DCs via lymph into lymphatic tissues.

Our findings emphasize the importance of DC-SIGN in the unique function of DCs, both in mediating naïve T cell interactions through ICAM-3, and as a rolling receptor that mediates the DC-specific ICAM-2-dependent migration processes. The fact that DC-SIGN functions as a novel HIV-1 trans-receptor important in the initial capture of HIV-1 at the periphery and transport by DCs into lymphoid tissues, again demonstrates that the unique migratory capacity of DCs can be exploited for dissemination of viruses such as HIV (Geijtenbeek *et al.*, 2000b). We have found that DC-SIGN is involved in the migratory capacity of both blood-borne DC precursors and tissue DCs; this suggests that clinical strategies which target DC-SIGN will be successful both in restricting HIV-1 dissemination and pathogenesis, and in directing the migration of DCs to manipulate appropriate immune responses in autoimmunity and tumorigenic situations.

ACKNOWLEDGMENTS

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Chapter 6

The extracellular domain of CD83 inhibits dendritic cell-mediated T cell stimulation and binds to a ligand on dendritic cells

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ABSTRACT

CD83 is an immunoglobulin (Ig) superfamily member that is upregulated during the maturation of dendritic cells (DCs). It has been widely used as a marker for mature DCs, but its function is still unknown. To approach its potential functional role, we have expressed the extracellular Ig domain of human CD83 (hCD83ext) as a soluble protein. Using this tool we could show that immature as well as mature DCs bind to CD83. Since CD83 binds a ligand also expressed on immature DCs, which do not express CD83, indicates that binding is not a homophilic interaction. In addition, we demonstrate that hCD83ext interferes with DC maturation downmodulating the expression of CD80 and CD83, while no phenotypical effects were observed on T cells. Finally, we show that hCD83ext inhibits DC-dependent allogeneic and peptide-specific T cell proliferation in a concentration dependent manner *in vitro*. This is the first report regarding functional aspects of CD83 and the binding of CD83 to DCs.

INTRODUCTION

Dendritic cells (DCs) are the most potent APCs of the immune system. DC maturation, with its associated functional and phenotypic changes is crucial to this role. In their immature state, DCs reside in peripheral tissues. Upon antigen uptake and inflammatory and microbial stimuli, they start to migrate to the T cell areas of the peripheral lymph nodes, where as mature DCs, they express additional molecules that will lead to binding and stimulation of T cells, while losing their ability for antigen-capturing (Steinman, 1991; Banchereau & Steinman, 1998; Dieu *et al.*, 1998). In this respect, CD83 is the best known marker for mature DCs. Human CD83 (hCD83) is a 45-kD glycoprotein and member of the Ig superfamily (Zhou *et al.*, 1992; Kozlow *et al.*, 1993). Recently, also the cloning and biochemical characterization of the murine CD83 (mCD83) has been reported (Twist *et al.*, 1998; Berchtold *et al.*, 1999). mCD83 shares an amino acid identity of 63% with hCD83 suggesting a possibly conserved function.
The selective expression and upregulation together with costimulatory molecules like CD80 and CD86 suggests an important role of CD83 in the immune response (Zhou *et al.*, 1992; Weissman *et al.*, 1995; Zhou & Tedder, 1995; Zhou & Tedder, 1996). Although the precise function of CD83 is still unknown (no inhibitory antibody exists), we have recently shown that by interfering with the translocation of mRNA encoding CD83 and thus inhibiting CD83 protein synthesis, the T cell stimulation capacity of DCs was significantly reduced (Kruse *et al.*, 2000a). In addition, we demonstrated a selective downregulation of the surface expression of CD83 after the infection of mature DCs with Herpes simplex virus type 1 and a reduced ability to stimulate allogeneic T cells in mixed leukocyte reactions (MLRs; Kruse *et al.*, 2000b). These observations indicated, but did not prove a critical role for CD83.
Here we report the recombinant expression and purification of the extracellular domain of the hCD83 (hCD83ext). Interestingly, hCD83ext completely abrogates the DC-mediated primary allostimulation of T-cells *in vitro*. Furthermore, also the capacity to stimulate antigen specific T cells was inhibited by this soluble molecule. This is the first report describing a functional role for CD83 which will contribute to a better understanding of the DC biology in general and will hopefully lead to the development of new therapeutic strategies. Finally, we show that CD83 binds to immature as well as mature DCs.

MATERIALS AND METHODS

Cloning of hCD83ext

The extracellular domain of human CD83 (amino acids 23-128) was PCR-amplified using the following primers: sense- pGEX2ThCD83: 5`-TCCCC-CGGGAACGCCGGAGGTGAAGGTGGCT-3` and antisense-CD83extra: 5`-AATTAGAA-TTCTCAAATCTCCGCTCTGTATT-3`. The amplified fragment was subcloned into the SmaI and EcoRI sites of the expression vector pGEX2T (Amersham Pharmacia Biotech, Freiburg, Germany) resulting in the plasmid pGEX2ThCD83ext and transformed into the E.coli strain TOP10F` (Invitrogen, Groningen, The Netherlands). The correct insert was verified by sequencing.

Expression and purification of hCD83ext

The expression of hCD83ext was induced in Escherichia coli as described previously (Berchtold *et al.*, 1999). Briefly, the cells were then pelleted and resuspended in 10ml native buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.6 mM MnCl, 26 mM $MgCl_2$, 1μg/ml leupeptin, 1μg/ml

aprotinin, 1μg/ml DNAseI, pH 7.6) per 500 ml culture. 50 μg/ml lysozyme was also added. After 15-min incubation on ice the lysate was spun at 20,000 g. Protein purification: capture step: 40 ml supernatant were added to a GSTrap 5 ml column on an ÄKTA Explorer 10 system (Amersham Pharmacia Biotech). Binding buffer: PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.6). Elution buffer: 50 mM Tris-HCl, pH 8.0, with 5 mM reduced glutathione. Flow rate: 5 ml/min. Chromatographic procedure: 4CV (column volumes) binding buffer, 40 ml supernatant, 12CV binding buffer, 5CV elution buffer, 5CV 2N NaCl/PBS, pH 7.6, 5CV binding buffer. Intermediate purification steps: The GST-hCD83ext. containing elutions were dialysed against 50 mM 1-methyl-piperazine, 50 mM Bis-Tris , 25 mM Tris pH 9.5 (buffer A), and loaded onto source 15Q PE 4.6/100 anion exchange column on a ÄKTA Explorer 10 system. Proteins were separated by three different linear salt gradients: 16CV to target concentration 10% bufferB (buffer A/1N NaCl). 20CV to target concentration 50% buffer B. 10CV to target concentration 100% Buffer B. The GST-hCD83ext. containing elutions were dialysed against PBS pH 7.6. Then the GST-hCD83ext. fusion protein was incubated with Thrombin 20 U/ml at 22°C for 16h. To separate the hCD83ext protein from GST the elution was loaded onto prepacked glutathion sepharose 4B columns using the capture step buffer conditions. Under binding buffer conditions the flow through containing recombinant human CD83ext protein was collected. Polishing Step: finally a preparative gel filtration separation was performed loading the flow through onto a Superdex 75 (26/16) prep grade column on an ÄKTA Explorer 10 system, running buffer PBS, pH 7.6, flow rate 3 ml/min. The GST protein was used as a negative control in functional analyses.

Expression and purification of recombinant CD83-Fc-fusion protein

CD83-Fc was constructed by fusing the extracellular domain of CD83 with the Fc part of human IgG1. A PCR product of the extracellular domain of CD83 was amplified using Pfu polymerase (Promega, Mannheim, Germany). The CD83 sense primer contains a XhoI restriction site (5'-GCGGGGCTCGAGGCCACCATGTCGCGCGGCCTCCAGCTTCTGC) and the antisense primer a BglII restriction site (5'-CCCCGGAGATCTGCAGGGCATCCTGTCACTCTCA). PCR conditions were as follows: 2min 94°C; 35x (1 min 94°C, 1 min 56°C, 1 min 72°C); 10 min 72°C. The PCR product was purified using a PCR purification kit (Qiagen, Hilden, Germany). After digestion with XhoI and BglII the PCR product was cloned into the XhoI and BamHI sites of the pCDM7 vector (a gift from Dr. Kolanus, Genecenter, Munich, Germany) containing the Fc part of IgG1 and transformed into MC1061P3 bacteria. The construct was sequenced in order to exclude possible mutations (Sequiserve, Vaterstetten, Germany). 293-T cells, cultured in DMEM medium (Life Technologies, Karlsruhe, Germany) supplemented with 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin/streptomycin (Life Technologies), 1 mM sodium-pyruvat (Life Technologies) and 10% FBS (Dynacyte, Heidelberg, Germany), were transiently transfected with CDM7/CD83-Fc using LipofectAMINE™ reagent and OPTIMEM 1 medium (Life Technologies). 293-T cells were cultured 10 days in serum free medium (293 SFMII; Life Technologies). Secretion of recombinant CD83-Fc protein into the supernatant was verified by Western Blot analysis. The CD83-Fc fusion protein was purified by protein A-Sepharose affinity chromatography (Amersham Pharmacia Biotech, Freiburg, Germany).

Production of mAbs against human CD83

~ 50 μg of the hCD83ext-GST fusion protein were injected intraperitoneally and subcutaneously into LOU/C rats. After a two month interval a final boost with the antigen was given intraperitoneally and subcutaneously three days before fusion. Fusion of the myeloma cell line P3X63-Ag8.653 with the rat immune spleen cells was performed according to standard procedure. Hybridoma supernatants were tested in a solid-phase immuno assay using the hCD83ext-GST protein adsorbed to polystyrene microtiter plates. After incubation with culture supernatants for 1 h, bound mAbs were detected with peroxidase-labelled goat anti-rat IgG plus IgM antibodies (Dianova, Hamburg, Germany) and O-phenylenediamine as chromogen in the peroxidase reaction. An irrelevant GST-fusion protein served

as a negative control. Ig type of the monoclonal antibodies was determined using biotinylated anti-rat IgG subclass-specific monoclonal antibodies (American Type Culture Collection, Rockville, MA, USA). CD83-1G11 (rat IgG1) and CD83-4B5 (rat IgG2a) were used for Western blot analysis and FACS® analysis. Both detected specifically human CD83. Functional studies revealed that, like the commercially available anti-CD83 antibodies, these antibodies had no inhibitory activity.

Immunoblotting analyses
Western blot analyses of purified protein was performed as described previously (Kruse *et al.*, 2000a) using monoclonal anti-CD83 antibodies (CD83-1G11; CD83-4B5 both described in this paper; and anti-CD83 from Immunotech, Marseilles, France).

1-D NMR studies
1-D NMR studies were recorded with a sample of the hCD83ext using a Bruker AM 400 spectrometer at a temperature of 300K as described previously (Berchtold *et al.*, 1999).

Generation of Dendritic Cells
DCs were generated from PBMCs in the presence of GM-CSF and IL-4 as described previously (Kruse *et al.*, 2000a). For the final DC maturation the medium containing 1% human plasma, GM-CSF and IL-4 was supplemented with IL-1β (1 ng/ml), TNFα (1.25 ng/ml), and prostaglandin E2 (0.5 mg/ml) (maturation mix).

Plate adhesion assay
A 96-well flat-bottom plat (Maxi Sorp, Nunc, Roskilde, Denmark) was precoated with 50 μl goat anti-human Fc-specific F(ab`)2 (4 μg/ml, Jackson Immuno Research Laboratories, Inc, West Cove, PA, USA) or with 50 μl rat anti-human GST (1 μg/ml) for 1 h at 37°C and blocked with 1% BSA in Tris-sodium buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$) for 30 min at 37°C. Subsequently, the plate was incubated with 500 ng/ml intercellular adhesion molecule (ICAM)-1-Fc (13), 500 ng/ml CD83-Fc. 40,000 cells/well were labeled in PBS with Calcein-AM (25 μg/10^7 cells per milliliter; Molecular Probes, Eugene, OR, USA) for 30 min at 37°C. Labeled cells were washed and preincubated for 10 min with different concentrations of recombinant hCD83ext or medium at RT. Cells were allowed to adhere for 45 min at 37°C. Adherent cells were lysed with 100 μl lysis buffer (50 mM Tris, 0.1% SDS), and the fluorescence was quantified using a Cytofluor II (Perseptive Biosystems, Farmingham, MA, USA) or a Victor (Wallac, Turku, Finland) fluorometer. Results are expressed as the mean percentage of cells binding from triplicate wells.

Coating of beads with CD83-Fc
Streptavidin-coated TransFluoSpheres (488/645 nm; 1,8 µm; Molecular Probes, Leiden, The Netherlands) were generated as described previously (Geijtenbeek *et al.*, 1999). The streptavidin-coated beads (15 μl) were incubated with biotinylated goat anti-human anti-Fc $F(ab`)_2$ fragments (33 μg/ml) in 0.3 ml PBS, 0.5%BSA for 2 h at 37°C. The beads were washed once with PBS, 0.5% BSA, and incubated with CD83-Fc (500 ng/ml) in 500 µl PBS, 0.5% BSA overnight at 4°C. The binding efficiency was determined by FACS® analysis.

Flow cytometric analysis
Phenotypic analysis of cells or beads were performed by flow cytometry using saturating concentrations of the following mAbs: CD83 (CD83-1G11; CD83-4B5 both described in this paper and anti-CD83 from Immunotech, Marseille, France); CD80 (Immunotech); CD14 and CD86, MHC-I (Dianova, Hamburg, Germany); CD3 (Bio Research, Kaumberg, Austria), CD4, CD8, CD45RO, and CD45RA (Dako, Glostrup, Denmark). The isotype controls IgG1a, IgG2a, and IgG2b (Becton

Dickinson, Heidelberg, Germany) were run in parallel. Cells were analyzed on a FACScanTM (Becton Dickinson). Nonviable cells were gated out on the basis of their light scatter properties.

Allogenic T cell proliferation

Human PBMCs were isolated from buffy coats and the T cell fraction was purified by rosetting with neuramidase-treated sheep RBCs as described by Bender et al. (Bender *et al.*, 1996). The CD4- and CD8-positive T cells were stimulated at different ratios with mature allogeneic DCs. DCs were treated with different concentrations of hCD83ext. GST or BSA was used as negative control (Bio-Rad Laboratories, Munich, Germany). CD83-Fc coated beads were also used for inhibitory studies. In these experiments uncoated beads were used as negative control. T cell proliferation was determined as described previously (Kruse *et al.*, 2000a).

Enzyme-linked immunospot assay

Assessment of the IFN-γ production by the influenza matrix peptide M1 specific CTL clone (C9) was performed as described previously (Strobel *et al.*, 2000). The HLA-A2.1 restricted M1 peptide (GILGFVFTL, amino acids 58-66) was used at a concentration of 10 µM. The number of spots forming cells was counted with a video imaging analysis system (Carl Zeiss Vision, Eching, Germany), using the KS enzyme-linked immunospot assay (ELISPOT) software version 4.1.143.

RESULTS

Recombinant protein expression

The extracellular domain of hCD83 (hCD83ext) was expressed in E.coli as a glutathione S-transferase (GST) fusion protein in sufficient quantities. For functional studies the fusion protein was cleaved by thrombin and only the hCD83ext was used, whereas GST served as a negative control. The correct expression of the protein was analyzed by silver staining and Western blot analysis (Fig. 1A and B).

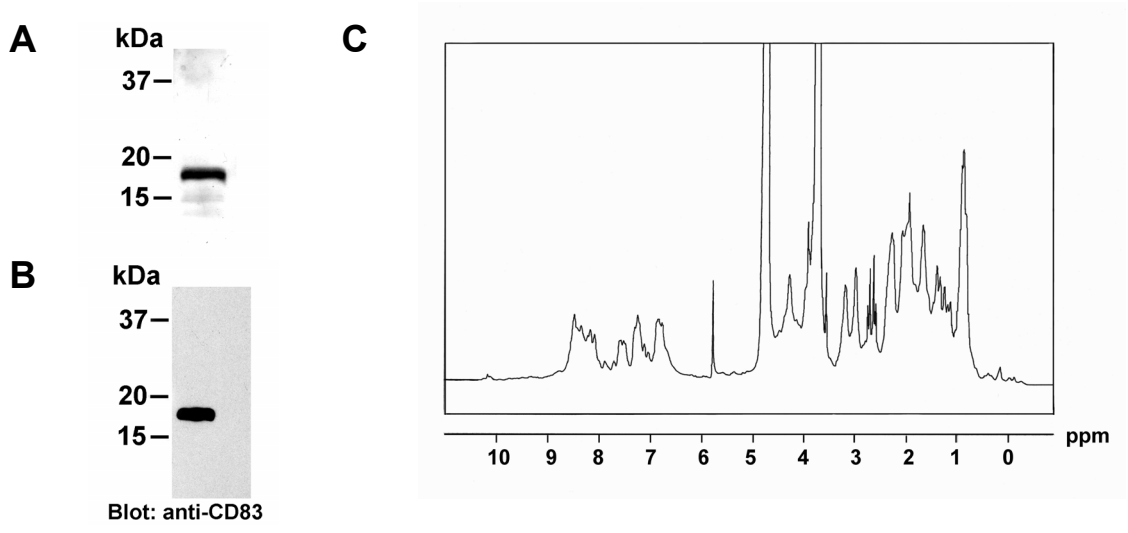

Figure 1. Biophysical analyses of recombinant hCD83ext. (A) hCD83ext was separated by SDS-PAGE and silver stained. (B) Western blot analysis of the blotted hCD83ext using monoclonal anti-CD83 antibodies and (C) one-dimensional NMR spectrum, showing that hCD83ext is folded.

Amino-terminal amino acid sequencing analyses further confirmed the correct identity of the purified protein (data not shown). To determine whether or not the recombinant protein was correctly folded, one-dimensional NMR studies were performed. The 1-D NMR spectrum of hCD83ext at 300K showed chemical dispersion typical of a structured protein (Fig. 1C). The presence of slowly exchanging amide resonances (~7-9 ppm) indicates that certain parts of the protein backbone are protected from solvent. Downfield-shifted a-CH resonances (~4.5-5.7 ppm) are indicative of β-structures. Upfield-shifted methyl resonances (s < 0.9 ppm) provide further evidence of the protein being folded. These NMR data strongly support that the recombinant expressed hCD83ext is structurally folded and relevant functional studies can be performed using this protein.

Human CD83 binds to immature and mature DCs

To establish the cell surface binding of human CD83 a standard plate adhesion assay was employed. As shown in Fig. 2 immature day 4 DCs as well as mature day 7-8 DCs (Fig. 2A and B, respectively) bind to human CD83. This binding could be inhibited in a concentration-dependent manner by the recombinant expressed extracellular domain of CD83 (hCD83ext). Binding to ICAM-1-Fc as a control could not be inhibited by hCD83ext. Uncoupled GST served as a negative control. The binding of ICAM-1 and GST was not specifically influenced by hCD83ext. In addition, as shown in Fig. 2C, binding of DCs to CD83 is concentration dependent. The specific binding was lost when decreasing amounts of CD83-Fc were used (Fig. 2C).

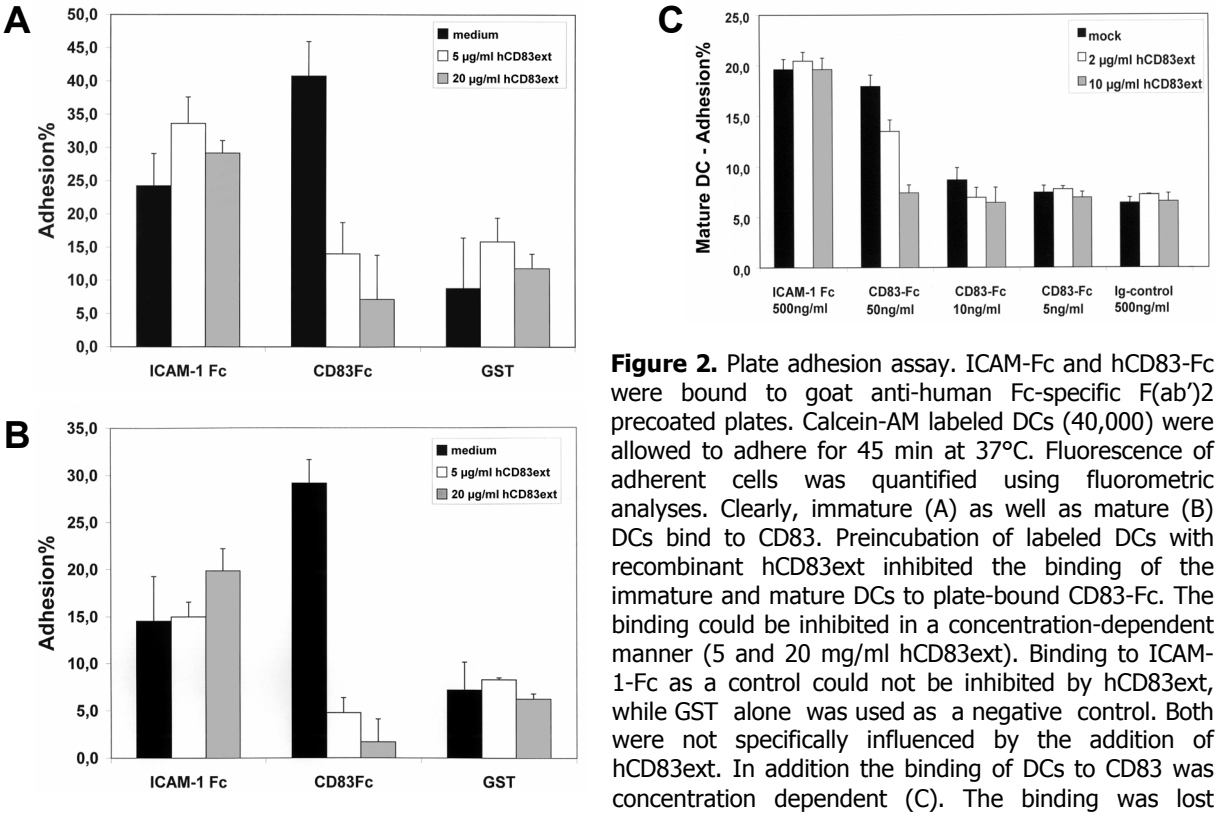

Figure 2. Plate adhesion assay. ICAM-Fc and hCD83-Fc were bound to goat anti-human Fc-specific F(ab')2 precoated plates. Calcein-AM labeled DCs (40,000) were allowed to adhere for 45 min at 37°C. Fluorescence of adherent cells was quantified using fluorometric analyses. Clearly, immature (A) as well as mature (B) DCs bind to CD83. Preincubation of labeled DCs with recombinant hCD83ext inhibited the binding of the immature and mature DCs to plate-bound CD83-Fc. The binding could be inhibited in a concentration-dependent manner (5 and 20 mg/ml hCD83ext). Binding to ICAM-1-Fc as a control could not be inhibited by hCD83ext, while GST alone was used as a negative control. Both were not specifically influenced by the addition of hCD83ext. In addition the binding of DCs to CD83 was concentration dependent (C). The binding was lost when decreasing amounts of CD83-Fc were used. In these experiments, Ig coated via goat anti-human-Fc, was used as negative control. The Ig binding was not influenced by hCD83ext. These experiments were performed five times using different donors for DC generation. This represents a typical experiment.

Interestingly, we could not demonstrate binding of naïve $CD4^+$ or $CD8^+$ T cells to CD83 (data not shown). From these data we conclude that immature and mature DCs bind specifically to CD83 and that the recombinant prokaryotic expressed hCD83ext could be used for further functional studies, indicating that glycosilation is not necessary. Furthermore, the fact that immature DC, which do not express CD83, also bind to CD83 implies that there is no homophilic interaction between CD83 molecules. Therefore, it is most likely a heterophilic binding reaction.

hCD83ext induces downmodulation of specific molecules on DCs

To analyze the influence of hCD83ext on the phenotype of DCs, FACS® analyses were performed on day 8 (Fig. 3). DCs can be fully matured with the use of a specific maturation cocktail composed of IL-1β, TNFα and PGE_2. Interestingly, when this maturation cocktail was administered to immature DCs on day 5 together with hCD83ext (4 μg/ml) and left until the final FACS® analyses on day 8, these cells revealed a clear reduction in CD80 (from 96% to 66%) and CD83 cell surface expression (96-30%), when compared with normally matured DCS (compare Fig. 3A with C). Thus, hCD83ext induces a reduction in DC maturation (see also increase of CD14-positive cells). In contrast, mature DCs, which where incubated with hCD83 for 24 h on day 7 and analyzed on day 8, showed only a minimal influence on CD80 expression (96-92%), while CD83 expression was also reduced (96-66%) (compare Fig. 3A with B). Interestingly, CD86 expression was not influenced at any time point by the administration of hCD83ext. Also MHC class I and II expression was not affected, neither in immature nor in mature DCs. Noteworthy, no significant phenotypical changes could be observed within T cell populations by the addition of hCD83ext (data not shown), indicating that CD83 affects primarily DCs.

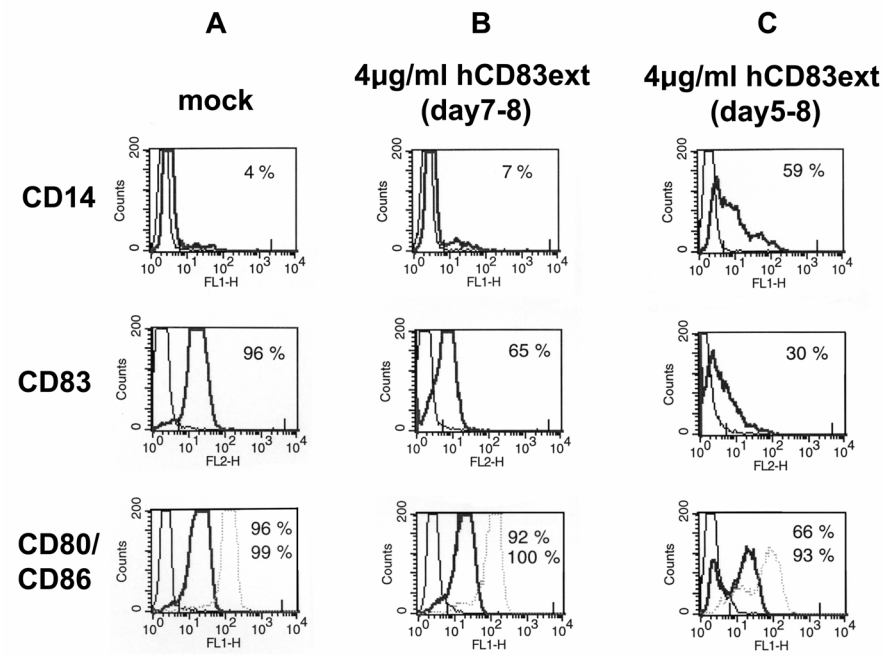

Figure 3. FACS® analyses of DCs. (A) Immature DCs were matured in the presence of the maturation cocktail from day 5-8 (= mock control for mature DC). (B) Immature DCs were matured in the presence of the maturation cocktail (day 5-8) and on day 7 hCD83ext was added for 24 h. (C) Immature DCs were incubated in the presence of the maturation cocktail in combination with hCD83 from day 5-8. On day 8 cells were washed and stained with the indicated antibodies and analyzed by FACS®.

hCD83ext inhibits allogeneic T cell proliferation

MLRs are a simple, yet very powerful tool to analyze the T cell stimulatory capacity of DCs. A typical feature of these MLR-assays is the formation of large DC-T cell clusters. Addition of hCD83ext at day 1 strongly inhibited the typical cell cluster formation of DCs and proliferating T cells, whereas GST had no influence (data not shown). As shown in Fig. 4A hCD83ext strongly inhibited [^{3}H]thymidine incorporation in a dose-dependent manner. GST, which was expressed and purified in the same way as hCD83ext, was used as negative control and had, like BSA, no influence on T cell proliferation. Also the CD83-Fc molecule (expressed in a eukaryotic system) coupled to beads inhibited T-cell proliferation (Fig. 4B). These findings indicate again that CD83-glycosylation is not a prerequisite, since the prokaryotic expressed hCD83ext also inhibits T cell proliferation.

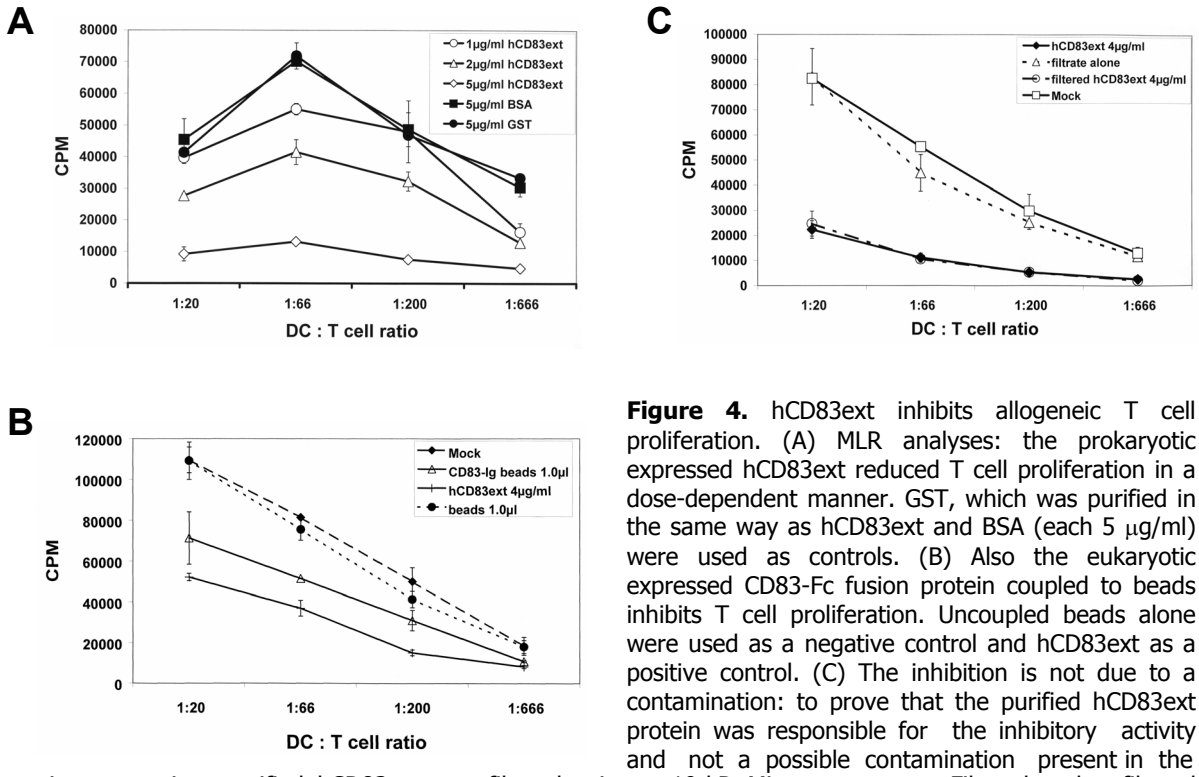

Figure 4. hCD83ext inhibits allogeneic T cell proliferation. (A) MLR analyses: the prokaryotic expressed hCD83ext reduced T cell proliferation in a dose-dependent manner. GST, which was purified in the same way as hCD83ext and BSA (each 5 µg/ml) were used as controls. (B) Also the eukaryotic expressed CD83-Fc fusion protein coupled to beads inhibits T cell proliferation. Uncoupled beads alone were used as a negative control and hCD83ext as a positive control. (C) The inhibition is not due to a contamination: to prove that the purified hCD83ext protein was responsible for the inhibitory activity and not a possible contamination present in the protein preparation, purified hCD83ext was filtered using a 10-kD Microcon system. Filtered and unfiltered hCD83ext exhibit comparable inhibitory effects, whereas cells which were treated with the filtrate alone showed no inhibition and the stimulation was comparable to mock treated cells. All experiments were performed at least three times. Data presented here represent a typical experiment.

To prove that the inhibitory effects of hCD83ext are not due to cytotoxic effects, day 5 DCs were incubated for 24 h with 5 µg/ml hCD83ext together with the final maturation mix. Then, hCD83ext was washed out, new maturation mix was added and the allostimulatory capacity of these cells was determined. Interestingly, T cell proliferation was not inhibited when hCD83ext has been washed out (data not shown). These data clearly show that hCD83ext is not cytotoxic and indicate that CD83ext must be present during the experiment to exhibit its inhibitory function.

To further demonstrate that the purified protein itself was responsible for the inhibitory activity and not a possible contamination present in the protein preparation, purified hCD83ext was filtered using a 10-kD Microcon system. Then, the filtered protein as well as the filtrate were tested (Fig. 4C). These experiments clearly show that the filtered hCD83ext protein was responsible for the inhibitory effects, while the filtrate had no influence on the T cell proliferation.
To demonstrate that hCD83ext had no toxic effects on T cell level, cell viability was determined using the trypan blue exclusion method. T cells were incubated with hCD83ext (4 μg/ml) for 4 days and compared with control cells (incubated with 4 μg/ml BSA). No significant decrease in T cell viability, after incubation with inhibitory concentrations of hCD83ext were detected (data not shown). Therefore, T cell death seems not responsible for the reduced proliferation.

hCD83 inhibits proliferation of influenza virus specific CTL

The ELISPOT assay is a very useful tool to analyze DC-mediated proliferation of specific CTLs, assessing the cytokine release at a single cell level. Furthermore, it can also be used for direct ex vivo quantification of peptide-reactive T cell responses. In this study we used the influenza matrix peptide M1 specific CTL clone C9 (Bednarek *et al.*, 1991). HLA-A2.1 positive DCs were incubated with the M1-peptide and then cocultivated with different numbers of CTLs. A computer-assisted video image analysis system was used for the evaluation of the experiments (Herr *et al.*, 1997). Again, increasing amounts of hCD83ext resulted in a clear reduction of CTL specific responses (Fig. 5), indicating that the interaction between DCs and peptide specific T cells is inhibited.

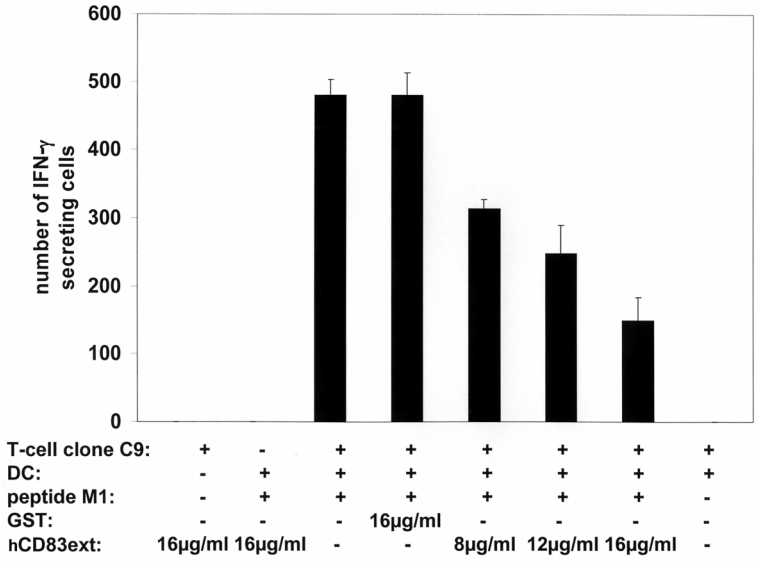

Figure 5. hCD83ext inhibits proliferation of influenza matrix peptide M1-specific CTL clone. DCs were loaded with influenza matrix peptide M1 and used to assess the stimulation (IFN-γ production) of the M1-specific CTL clone C9. Number of IFN-γ-producing spot are shown on the y-axis. Increasing concentrations of hCD83ext clearly reduced the number of spot forming cells. GST, which was purified in the same way as hCD83ext was used a negative control. Three independent experiments were performed. The data presented represent a typical experiment.

Secondary stimulation of hCD83ext treated T cells

To investigate whether or not the proliferation arrest of hCD83ext treated T cells can be overcome, cells from the primary MLRs, which have been either incubated with or without hCD83ext, were stimulated for a second time period of 4 days without the addition of hCD83ext. For this, half of the medium was discharged and new medium containing IL-2 at a final concentration of 500 U/ml or without IL-2 was added to the cultures. After an additional incubation of 3 days, cells were pulsed and T cell proliferation was determined (Fig. 6A). Interestingly, T cells which were strongly inhibited by hCD83ext in the primary MLRs could be restimulated with IL-2. The proliferation was comparable to that obtained by untreated cells in the primary MLRs. These data clearly show that the proliferation arrest in T cells, caused by hCD83ext, is not due to an irreversible toxic effect. In fact proliferation can be restored using IL-2. Furthermore, to investigate this phenomenon in a more physiological setting, T cells which were inhibited with hCD83ext in the primary MLRs, were restimulated with allogeneic DCs, instead of IL-2 (Fig. 6B). Again, arrested T cells could be restimulated with DCs, reaching stimulation levels comparable to those obtained with untreated cells in primary MLRs. Thus the inhibitory effect of hCD83ext is reversible and is not mediated via T cell anergy.

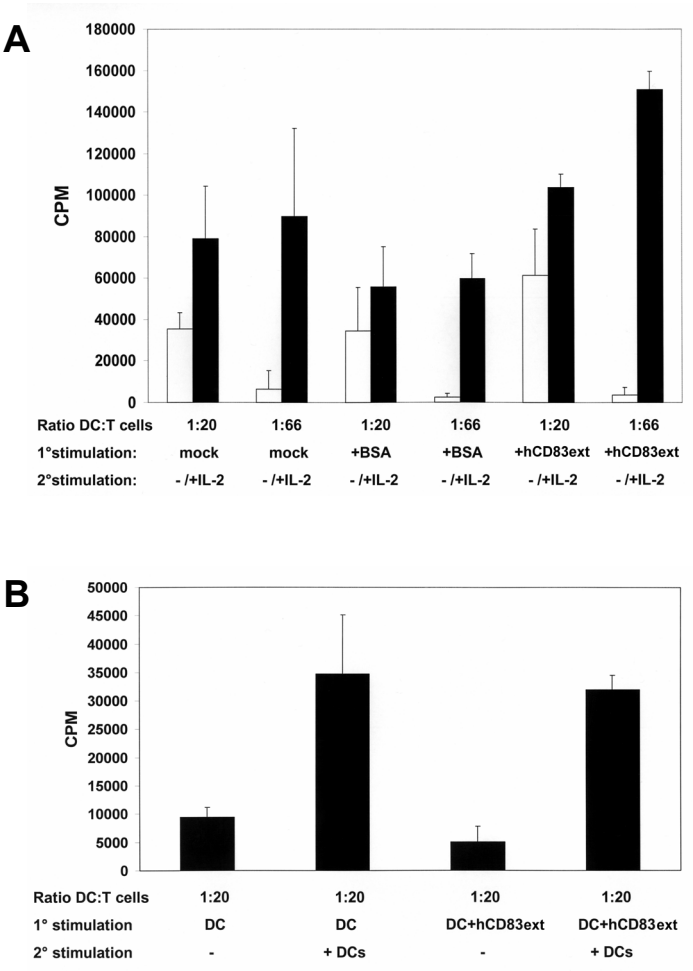

Figure 6. Proliferation arrest of hCD83ext treated T cells can be restored. (A) hCD83ext-treated T cells derived from the primary MLRs, which have been either treated with or without hCD83ext, were stimulated for a second time period of 4 days without the addition of hCD83ext. Medium containing IL-2 or medium without IL-2 was added to the cultures. After a 3-days incubation period proliferation was determined. T cells which were strongly inhibited by hCD83ext in the primary MLRs could be restimulated with IL-2, clearly showing that the proliferation arrest, caused by hCD83ext in the primary MLRs, is not due to an irreversible toxic effect. (B) T cells which were inhibited with hCD83ext (4 μg/ml) in the primary MLRs (ratio DC:T cell = 1:20) , were restimulated with allogeneic DC (ratio 1:20). Arrested T cells could be restimulated with DCs, reaching stimulation levels comparable to those obtained with untreated cells in primary MLRs. Thus, the inhibitory function of hCD83ext is reversible. Experiments were performed at least three times. The data presented here represent a typical experiment.

DISCUSSION

DCs act as nature's adjuvant in inducing T cell-mediated immunity. To understand the biological properties of DCs it is vital to gain further knowledge regarding the role of DC-specific proteins on a molecular level. Here, we provide for the first time evidence that CD83, the best known cell surface marker for mature DCs, has also a biological function. During DC maturation CD83 is rapidly upregulated together with the costimulatory molecules CD80 and CD86. These molecules provide the so-called second signal for the T cell activation (Schwartz, 1992). Multiple studies have shown that allostimulation can be prevented by blocking the CD28-mediated costimulatory signals using either anti-CD80/CD86 mAbs or the fusion protein CTLA4Ig (Boussiotis *et al.*, 1994; Lenschow *et al.*, 1995; Akalin *et al.*, 1996; Larsen *et al.*, 1996; Newell *et al.*, 1999). As a result this blockage of signal two leads to T cell anergy.

Although CD83 is strongly upregulated on mature DCs the function of this molecule has been unknown to date. To elucidate the mode of action of CD83 the extracellular Ig domain of human CD83 was expressed as a recombinant protein and used for functional studies *in vitro*. 1D-NMR data strongly support that the recombinant hCD83ext is correctly folded and therefore the suppressive effects observed in the T cell assays are due to a functionally active molecule. In addition, a CD83-Fc molecule, expressed in a eukaryotic system was used.

Using plate adhesion analyses we could show for the first time that immature and mature DCs bind to CD83. The specificity of the CD83-binding to DCs could be demonstrated by the fact that the soluble extracellular hCD83ext domain inhibited the binding in a concentration-dependent manner (Fig. 2 A-C). Furthermore, the binding of DCs to CD83 itself is also concentration dependent. The binding was lost when decreasing amounts of CD83 were used (Fig. 2C). These findings are highly relevant for several aspects: (i) since hCD83ext, which was expressed in a prokaryotic system, inhibits the binding of the eukaryotic expressed CD83-Fc underlines the fact that glycosilation is not necessary for CD83 binding to its ligand; (ii) the prokaryotic expressed hCD83ext protein is functionally active; (iii) the fact that CD83 binds also to immature DCs, which do not express CD83, indicates that there is no homophilic interaction between CD83 molecules. These findings rather suggest the existence of a heterophilic binding.

Interestingly, immature DCs which were incubated with soluble hCD83ext could not be fully matured anymore. Even in the presence of the potent maturation cocktail composed of IL-1β, TNFα, and PGE_2 the maturation was blocked. hCD83ext treatment lead to the specific downmodulation of CD80, a crucial costimulatory molecule and of CD83 itself. This suggests that soluble hCD83ext induces a maturation block, which could be a regulatory mechanism in order to control immune responses. In this respect it is very interesting to know that a soluble form of CD83, like described for CD86 (Jeannin *et al.*, 2000), has been characterized *in vivo* and is detectable in normal human sera and is released from activated DC and B-cells (Hock *et al.*, 2001), underlying a possible important role in the regulation of immune responses. In addition, also mature DCs downmodulate CD83 from their cell surface when incubated with hCD83ext, indicating a regulatory mechanism also on mature DCs. The soluble molecule may either work by delivering a negative signal or blocking a positive signal that might be generated by interactions of maturing DCs. However, this hypothesis has to be proven in an experimental setting.

Furthermore, when mature DCs were incubated with soluble hCD83ext they completely lost their ability to stimulate allogeneic T cell responses. In addition, also CTL specific T cell responses were inhibited by hCD83ext. Again, the existence of a soluble form of CD83 *in vivo* (Hock *et al.*, 2001) is an additional support of our data regarding the inhibitory effects of hCD83ext.
Noteworthy, these effects are not due to cytotoxic effects on T cells. We demonstrate that T cells can be restimulated either with IL-2 or with allogeneic DC. This represents a novel mechanism which is not connected to T cell anergy, induced by the inhibition of the B7-CD28 costimulatory pathway (Boussiotis *et al.*, 1994). Since the inhibition mediated by hCD83ext is a competitive inhibition, hCD83ext has to be present throughout the experiment. Dilution of the hCD83ext concentration with medium alone allowed already the restimulation of T cells. Also the number of APCs is crucial. High numbers of DCs can only be blocked with correspondingly higher hCD83ext concentrations.
Considering recent results, it becomes clear that CD83 plays an important role in DC biology. Previously, we could show that inhibition of CD83 expression, by interfering with a specific RNA export pathway, leads to a clear reduction of DC-mediated T cell stimulation (Kruse *et al.*, 2000a). An additional indication, pointing towards the biological importance of CD83 is the fact that several viruses including herpes simplex virus type 1 and poxviruses downregulate CD83 (Kruse *et al.*, 2000; Jenne *et al.*, 2000b). Interestingly, these virus infected DCs are also inhibited in their allostimulatory capacity (Salio *et al.*, 1999; Engelmayer *et al.*, 1999; Kruse *et al.*, 2000b; Jenne *et al.*, 2000; Drillien *et al.*, 2000). Nevertheless, regarding the biological function of CD83, these reports provided only circumstantial evidence.
In contrast, here we report the identification of CD83 binding to DC and the characterization of a reversible inhibitor of DC mediated T cell stimulation, representing a new tool to explore DC biology and new therapeutic strategies. Future studies including the use of k.o. mice will help to elucidate the biological role of CD83 even in more detail. Further characterizations are also needed regarding the CD83 structure and the characterization of the ligand(s) present on DCs.

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Chapter 7

Discussion

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Migration plays a crucial role in the lifespan of Dendritic Cells (DCs) and T cells. The migration of immature DCs and activated T cells into peripheral tissues and of mature DCs and naïve T cells into the lymph nodes is essential to elicit a proper immune response. The migration processes are very dynamic and modulated by chemokines. In addition, they require a continuous attachment and detachment of cells, tightly regulated by adhesion receptors and cytoskeletal constraints. In this chapter, the role of integrins and other adhesion receptors, like DC-SIGN and CD83 in DC adhesion and migration will be discussed. It will become clear that the regulated expression of DC-SIGN on DCs and integrins on DCs and T cells, the activation state of the adhesion molecules, the cytoskeletal constraint, and the maturation state of the cells determine the adhesive and migratory properties of the cells.

INTEGRINS

β1 integrins are involved in the interaction of immature as well as mature DCs with fibronectin. Whereas both DC-subtypes do express the α4 chain, they preferentially adhere to fibronectin through α5β1 (chapters 3 and 4). Most likely, the α5β1 molecule is more active than the α4β1 integrin on DCs and different cytoskeletal target molecules might be associated with the α5β1 than with the α4β1 integrin. The difference in receptor usage might be cell type dependent. Jancic and colleagues also show an α5β1-mediated adhesion of DCs to fibronectin. However, their human monocyte-derived DCs do not express α4 (Jancic *et al.*, 1998). Binding of DCs to a natural extracellular matrix produced by endothelial cells involves both α4β1 and α5β1 (Bianchi *et al.*, 2000). Adhesion and migration studies on fibronectin performed with other leukocytes also show a cell type dependent usage of either α4β1, α5β1, or of both α4β1 and α5β1 (Gismondi *et al.*, 1991; Hauzenberger *et al.*, 1994; Hauzenberger *et al.*, 1995; Crisa *et al.*, 1996; Peled *et al.*, 2000).
I observed that a higher percentage of immature DCs versus mature DCs binds to fibronectin. Moreover, adhesion of mature DCs, but not of immature DCs to fibronectin can be induced by a β1-activating antibody (chapter 4). Since no difference in β1 expression was observed, either a change in activation state of the molecule or a change in cell surface distribution of β1 accounts for this difference. Interestingly, the expression of the β1 activation epitope was reduced on mature DCs (chapter 4), indicating that the activation state of the α5β1 molecule determines the difference in adhesive and migratory behavior between both DC subtypes. The effect of the integrin activation state on adhesion and migration is also underscored in chapter 2, in which αLβ2 activation by the β2-activating antibody KIM185 resulted in an increased adhesion of HSB-2 T cells and as a result a decreased migration on ICAM-1 coatings. Moreover, I showed that ligand concentrations were critical in these processes; whereas adhesion increased with enhanced ligand concentration, only intermediate ligand concentrations did facilitate migration. Similar findings are observed by Palecek and colleagues, who showed that cells expressing α5β1 or αIIbβ3 have a biphasic migration pattern on fibronectin and fibrinogen respectively (Palecek *et al.*, 1997).

Signaling is required to modulate integrin activity. Both the affinity and avidity changes of integrins required to bind ligand can be regulated by inside-out signaling (van Kooyk & Figdor, 2000). Many modulators are involved in this activation process, including phorbol esters, G protein coupled signaling, particular antibodies, or divalent cations (Rothlein & Springer, 1986; Gailit & Ruoslahti, 1988; Danilov & Juliano, 1989; Shimizu *et al.*, 1990; van de Wiel-van Kemenade *et al.*, 1992; Arroyo *et al.*, 1992; Constantin *et al.*, 2000; Alon & Feigelson, 2002). Binding and rolling studies of $\alpha L\beta 2$ expressed in different cell types pointed at the importance of the cellular microenvironment in these processes; $\alpha L2$ expressed in K562 does facilitate rolling on ICAM-1 coatings, whereas $\alpha L\beta 2$-mediated rolling is not observed in PBL and Jurkat cells (Sigal *et al.*, 2000). Likewise, the regulation of integrin activity might be different in immature DCs and mature DCs. Several proteins that may account for this difference are discussed below. DCs express $\beta 1$ and $\beta 2$ integrins, which can both associate with cytoskeletal proteins. Here, I will focus on cytoskeletal proteins which can bind to $\beta 1$ integrins (Fig. 1), in particular $\alpha 5\beta 1$, since I observed that this integrin mediates differences in adhesion to fibronectin for immature and mature DCs.

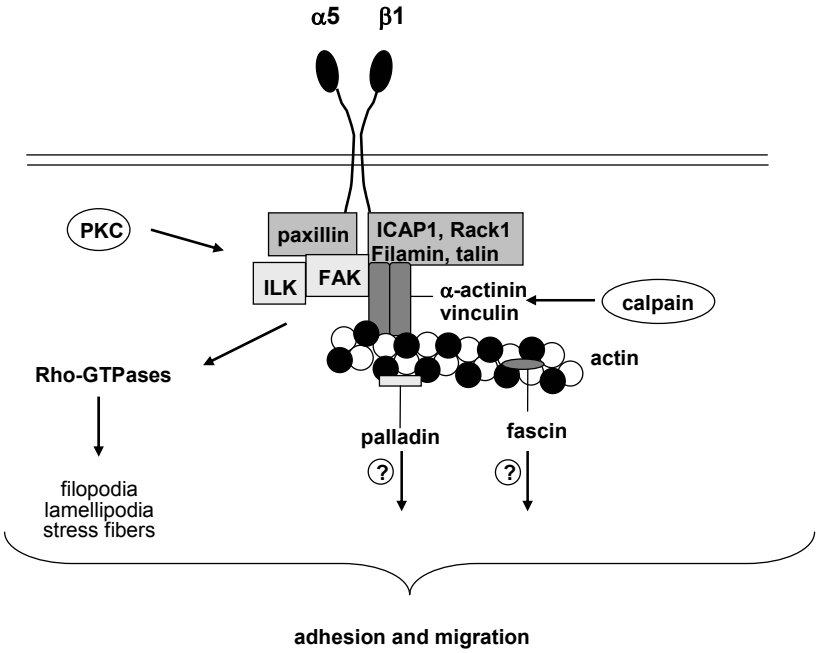

Figure 1. Overview of cytoskeletal and signaling proteins that link the $\alpha 5\beta 1$ integrin to the actin cytoskeleton. The actin-binding proteins talin, α-actinin, and filamin link integrins to the cytoskeleton, either direct or indirect via vinculin. The enzyme calpain cleaves these actin-bindings proteins from the integrin, resulting in freely mobile integrins. Integrins can stimulate Rho-GTPases via the signaling molecules FAK and ILK, but also via ICAP-1. Rho-GTPases are signal transducers that orchestrate cytoskeletal functions. PKC inhibits actin polymerization. In addition it phosphorylates proteins, like Rack1 and paxillin, and increases intracellular calcium levels. Calcium can subsequently activate proteases like calpain. The complex formed by these cytoskeletal proteins and integrin regulates adhesion and migration. The actin-bundling protein fascin and the microfilament-associated protein palladin are both upregulated upon DC maturation. The role of these proteins in DC adhesion and migration is currently unknown.

Role of actin cytoskeleton on β1 integrin activation

The actin cytoskeleton plays a pivotal role in cell adhesion and migration, as high concentrations of actin-disrupting agents completely abolish these processes. Filamentous actin and actin-associated proteins can form complex structures inducing membrane remodeling, such as filopodia, microspikes, lamellipodia, and stress fibers, which are involved in adhesion and migration (reviewed in Mitchison & Cramer, 1996). Interestingly, maturation of DCs results in rearrangement of the cytoskeletal organization. For instance, DC maturation results in a lack of podosome formation, resulting in a loss of organized vinculin and F-actin structures (chapter 4; Burns *et al.*, 2001). In podosomes, tyrosine-phosphorylated proteins and actin-related proteins (Arp) colocalize with F-actin as do WASp proteins (Burns *et al.*, 2001). In contrast to Burns and colleagues, who suggested a relation between podosome formation and cell adhesion, we showed a highly migratory behavior of mature DCs, which lack podosomes. Since mature DC migration was mediated by transient interactions with the matrix molecule fibronectin, we tentatively suggest that podosome formation is related to more amoeboid crawling.

An additional cytoskeletal protein, which is upregulated during DC maturation, is the actin-bundling protein fascin. Fascin plays an important role in dendrite formation, hinting to a role for fascin in DC migration (Mosialos *et al.*, 1996; Ross *et al.*, 1998; Ross *et al.*, 2000). Another example of a protein, which is induced upon DC maturation, is the microfilament-associated protein palladin. In immature DCs, palladin is expressed in actin-containing podosomes, whereas in mature DCs it is localized along actin filaments (Mykkanen *et al.*, 2001).

This maturation-induced rearrangement might regulate the binding of these cytoskeletal proteins to the β1 integrins. Actin-binding proteins that link β1 integrins to the cytoskeleton are talin (Horwitz *et al.*, 1986), α-actinin (Otey & Burridge, 1990), and filamin (Loo *et al.*, 1998). The β1 chain binds talin and α-actinin either direct or via other proteins, such as vinculin (reviewed in Geiger *et al.*, 2001). Point mutations in the β1 cytoplasmic chain lead to disruption of talin binding to the β1 integrin resulting in a decreased cell spreading (Kaapa *et al.*, 1999). Although the interaction between talin and the β1 integrin is necessary, it is not sufficient for integrin-mediated cell spreading and targeting of integrins to focal adhesions (Kaapa *et al.*, 1999). Both talin and α-actinin colocalize with integrins in focal adhesions, whereas filamin localizes to the cortical actin cytoskeleton and along stress fibers. Filamin also functions as an adapter protein for several signaling proteins that regulate cytoskeletal dynamics.

Other proteins that regulate β1 integrin activity are paxillin (Schaller *et al.*, 1995) and calpain (Rock *et al.*, 1997). Paxillin mediates bridges between α4β1 and the actin cytoskeleton, but does not link α5 to the cytoskeletal constraint (Liu *et al.*, 1999). However, loss of α5β1-mediated monocyte adhesion to fibronectin induced by interferon-β and interferon-γ is correlated with changes in actin and paxillin cytoskeleton (Surin *et al.*, 2000). Calpain regulates the β1 integrin-mediated T cell adhesion and spreading (Rock *et al.*, 1997). Talin, filamin, and α-actinin are identified as potential proteolysis targets for calpain, resulting in freely mobile integrin. Deactivation of calpain inhibits adhesion and cell spreading of T cells on fibronectin. In addition, calpain regulates cell migration by supporting the dissociation of integrins from the cytoskeleton at the end of the cell reducing the retraction of the cell's rear (Huttenlocher *et al.*, 1997). As immature DCs spread on fibronectin and have more active β1,

it is very likely that calpain is active in these cells. Inhibition experiments blocking calpain activity should confirm this hypothesis.
Since all those cytoskeletal proteins bind the cytoplasmic tail of β1, it would be very interesting to study the colocalization of the discussed proteins with the α5β1 integrin in our DC culture setting.
It is important to note that under physiological circumstances cells display 3D-matrix adhesions resulting in a distinct cytoskeletal organization of proteins than on 2D coatings. For example α5 in fibroblasts placed on tissue- or cell-derived 3D matrices colocalizes with paxillin, vinculin, focal adhesion kinase (FAK), phosphotyrosine, α-actinin, and activated β1 integrin, whereas this is not demonstrated on 2D substrates (Cukierman *et al.*, 2001). Another difference observed between 2D and 3D matrix interactions are the integrins used for migration. For instance, the migration of T cells in collagen matrices is reduced by an activating β1 antibody, whereas it is not inhibited by a combination of β1, β2, β3, and αv blocking antibodies, suggesting that the migration is β1 integrin independent, which contrasts with 2D systems (Friedl *et al.*, 1998). 3D matrix interactions *in vivo* may also be distinct from 3D interactions *in vitro*. *In vivo* imaging of T cells in lymph nodes indicates different migration speeds compared with *in vitro* studies in collagen matrix models (Friedl *et al.*, 1994; Miller *et al.*, 2002).

Role of signaling molecules on β1 integrin activation

Next to a distinct organized actin cytoskeleton, a difference in signaling proteins that bind to the β1 integrin cytoplasmic domains might be responsible for the distinct adhesive properties of immature versus mature DCs. These proteins are focal adhesion kinase (FAK) (Schaller *et al.*. 1995), integrin-linked kinase (ILK) (Hannigan *et al.*, 1996), and receptor for activated protein kinase C (Rack1) (Liliental & Chang, 1998). ILK localizes to focal adhesions and has been implicated in the regulation of cell adhesion and cell migration. The interaction between Rack1 and integrins requires stimulation of the cell with phorbol esters, indicating that this interaction is regulated. FAK is an adapter protein with kinase activity, which localizes to integrins at the cell substratum contact sites and its activation state is regulated by cell-ECM interactions (reviewed by Schlaepfer & Hunter, 1998). In an *in vitro* DC differentiation model, transformation of DC progenitor cells from chicken bone marrow with a v-Rel estrogen receptor (ER) fusion protein v-RelER can result in highly motile v-relER DCs (Boehmelt *et al.*, 1995). In addition, this differentiation reduces cell-extracellular matrix interactions (Madruga *et al.*, 1999). These v-relER DCs exhibit a polarized pattern of actin and vimentin, which is also observed in human DCs derived from $CD34^+$ cells. Moreover, the expression of FAK was reduced upon differentiation. However, downregulation of FAK is apparently not essential in motility mechanisms since ectopic FAK expression in vRelER DCs does not affect their adhesion and migration. This suggests that FAK is most probably not responsible for the difference in adhesion between immature and mature DCs.
Another protein, which binds the β1 tail of integrins but is not directly involved in signaling, is ICAP-1 (integrin-cytoplasmic-domain-associated protein 1) (Chang *et al.*, 1997). ICAP-1 overexpressing COS cells display an enhanced β1-mediated migration. Upon cell-fibronectin interactions, ICAP-1 becomes phosphorylated. This may imply that immature DCs have more phosphorylated ICAP-1 than mature DCs. The role of ILK, FAK, Rack-1, and ICAP-1 in β1

integrin-mediated adhesion in immature versus mature DCs needs to be studied in future experiments.

Role Rho-GTPases and PKC on β1 integrin activation

Rho-GTPases are signal transducers orchestrating cytoskeletal functions (reviewed in Etienne-Manneville & Hall, 2002; Keenan & Kelleher, 1998). The regulation of actin polymerization is orchestrated by Rho/Rac/Cdc42 proteins. Rho proteins are involved in the formation of stress fibers, while Rac proteins mediate ruffling and lamellipodia formation, and Cdc42 regulates filopodium formation (reviewed in Takai *et al.*, 2001). Rho-family proteins also control podosome assembly and localization in immature DCs (Burns *et al.*, 2001). Rho activation leads to tail detachment of migrating monocytes, neutrophils, and eosinophils by stimulation of actomyosin filament contraction through negative regulation of integrin-mediated adhesion at the rear of the cell (Worthylake *et al.*, 2001; Alblas *et al.*, 2001). Worthylake and colleagues propose a model in which RhoA is required to downregulate integrin adhesions at the rear of the cells to allow tail retraction. Recently, Nischarin a novel intracellular protein that binds α5 has been described which affects Rho-family GTPases signaling resulting in inhibition of cell motility and alterations in actin filament organization (Alahari, *et al.*, 2000). In mice, Nischarin is expressed in brain and kidney; lower levels were observed in heart, liver, lung, and skeletal muscle, whereas no expression was seen in spleen and testis. So far, no data exist for Nischarin expression in DCs.
PKCs are involved in the activation of integrins, and subsequently in adhesion and migration (Rigot *et al.*, 1998; Ng *et al.*, 1999; Sun & Rotenberg, 1999). PKCε regulates β1 integrin-mediated migration through internal trafficking of these receptors through motile cells (Ivaska *et al.*, 2002). Upon PKC inhibition, PKCε and β1 are accumulated in tetraspanin CD81-positive intracellular vesicles, resulting in reduced cell migration.

Role of integrin-associated molecules on integrin activation

Next to the regulation of this internal traffic step of integrins, the association of tetraspanins with integrins is predicted to influence cell migration by controlling spatial organization of membrane complexes or modulating integrin signaling (Berditchevski, 2001). However, no data are available showing the influence on integrin conformation. α5β1 can associate with CD9, CD81, CD82, and CD151 (reviewed in Berditchevski, 2001) in different cell types. Transfection of CD9 into CHO cells resulted in association with α5β1 and F-actin, α-actinin, FAK, and subsequent affected adhesion to fibronectin (Cook *et al.*, 2002). We observed no colocalization of CD9 and α5β1 in DCs (unpublished results), indicating that CD9 may not be involved in DC adhesion to fibronectin.
A transmembrane proteoglycan which binds to α5β1 is syndecan-4. α5β1 requires the cell surface proteoglycan syndecan-4 for focal adhesion formation and migration (Woods & Couchman, 2001; Bass & Humphries, 2002). The α5β1/syndecan-4 complex directly activates PKCα, which in turn might stimulate Rho GTPases (Mostafavi-Pour *et al.*, 2003).
It remains to be determined if tetraspanins or syndecan-4 are expressed in cultured DCs, and if so, colocalize with α5β1 in immature and mature DCs and whether such associations might regulate adhesion in DCs.

Role of chemokine and lipid receptor signaling and integrin cross-talk on integrin activation

Integrin activation can also be accomplished by signaling via chemokine receptors. Immobilized chemokines on endothelium can induce arrest, but also earlier integrin-mediated capture of lymphocytes (Campbell *et al.*, 1998; Grabovsky *et al.*, 2000; Constantin *et al.*, 2000). In addition, chemokines also induce integrin-mediated T cell adhesion to ECM molecules, like fibronectin (Clissi *et al.*, 2000). Increasing evidence supports leukocyte migration by ligation of lipids, like lysophosphatidic acid (LPA) with their respective receptors, ligation of hormones, like prostaglandin E2 (PGE_2) with their receptors coupled to the Gs proteins (EP receptors), or ligation of nucleotides, such as adenosine triphosphate (ATP) with purinergic receptors on DCs. Maturation of DCs via a maturation cocktail including PGE_2 induces DC migration (Luft *et al.*, 2002; Scandella *et al.*, 2002). Engagement of ATP with their receptors results in a migratory DC phenotype (la Sala *et al.*, 2002). Future studies should reveal whether lipid mediators and nucleotides are able to signal to integrins.

Signaling via other integrins can also regulate integrin-mediated adhesion. For example, adhesion of human T cells to ICAM-1 via $\alpha L\beta 2$ decreases the adhesion mediated via $\alpha 4\beta 1$ to VCAM-1 and fibronectin, but has less effect on $\alpha 5\beta 1$-mediated binding (van Kooyk *et al.*, 1993; Porter & Hogg, 1997). This latter cross talk may be important for the transition of adherent to a more migratory state of the cells.

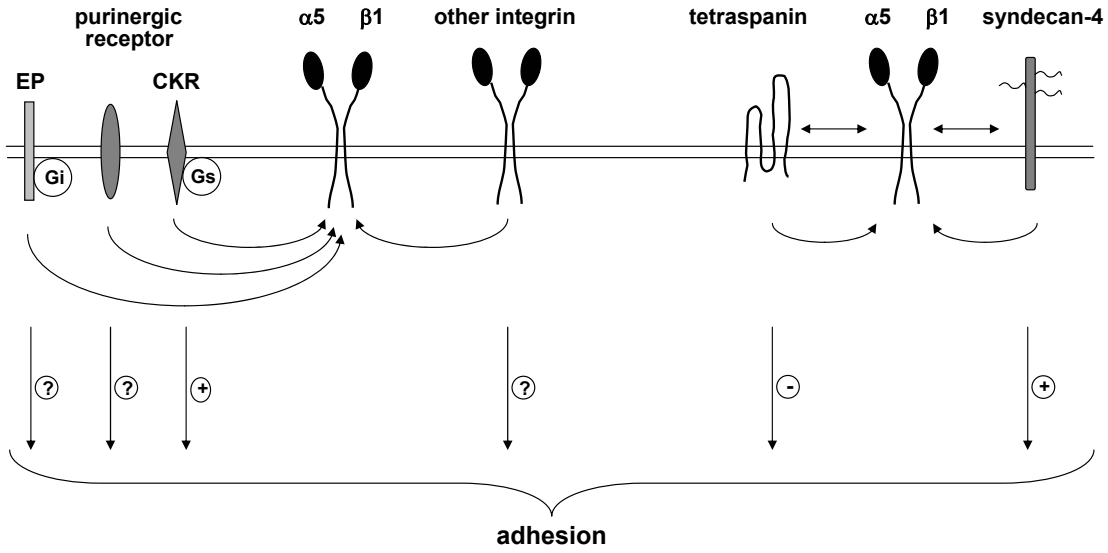

Figure 2. Overview of possible cross talk or associations between transmembrane receptors and the $\alpha 5\beta 1$ integrin. Upon chemokine binding chemokine receptors (CKR) activate $\alpha 5\beta 1$ resulting in enhanced adhesion to fibronectin. The activation of $\alpha 5\beta 1$ by cross talk via other integrins, the PGE_2 receptor (EP) or purinergic receptors is unknown as far. Tetraspanins and syndecan-4 can associate with $\alpha 5\beta 1$. Whether these molecules can modulate integrin conformation has to be investigated. Both tetraspanins and syndecans can bind extracellular matrix molecules. The association of the tetraspanin CD9 with $\alpha 5\beta 1$ results in decreased adhesion to the ECM, whereas the association between syndecan-4 and $\alpha 5\beta 1$ is required for focal adhesion formation.

Fig. 2 gives an overview of receptors, which might affect integrin activity. Future investigations should shed light on the possible existence of such signaling routes. From this summary of proteins that regulate β1 integrin activity it becomes clear that many factors orchestrate integrin-mediated adhesion and migration. Not much is known about the role of these proteins in DCs. It would be very interesting to investigate cytoskeletal rearrangements and α5β1 integrin-linked associations in immature versus mature DCs to unravel the distinct adhesive and migratory properties between those subtypes.

DC-SIGN

DC-SIGN was originally identified from a placental cDNA library as a C-type lectin binding the HIV-1 envelope glycoprotein gp120 (Curtis *et al.*, 1992). Later, it was found to be a DC-specific adhesion receptor (Geijtenbeek *et al.*, 2000a; Geijtenbeek *et al.*, 2000b). DC-SIGN binds to the Ig-superfamily members ICAM-2 and ICAM-3 under static, and in case of ICAM-2 also under flow conditions (chapter 5). Interestingly, DC-SIGN also supports tethering and rolling interactions on ICAM-2. I want to stress that the rolling capacity of DC-SIGN is investigated in transfected K562 cells and not in a DC environment. As discussed above, αLβ2 transfected in K562 also mediates rolling interactions, whereas wild-type αLβ2 in peripheral blood lymphocytes and Jurkat cells does not mediate rolling (Sigal *et al.*, 2000). Sigal and colleagues hypothesized that the difference in rolling capacity of αLβ2 depends on the restriction in αLβ2-mediated adhesion strengthening, which is cell type dependent. Therefore, it would be interesting to test the involvement of DC-SIGN expressed on DCs in rolling, *in vitro* on ICAM-2 coatings and endothelium, or *in vivo* by intravital microscopy, both in combination with DC-SIGN blocking antibodies or using DCs treated with RNAi of DC-SIGN to knock out DC-SIGN expression.
DC-SIGN is also involved in binding and transendothelial migration of DCs, to resting as well as activated endothelium (chapter 5). In contrast to Geijtenbeek and colleagues who found DC-SIGN expression on $CD34^+$-derived DCs, Nguyen and colleagues showed that their $CD34^+$-derived DCs lack DC-SIGN and adhere to resting dermal microvascular endothelium via αLβ2, αMβ2, and CD36 (Geijtenbeek *et al.*, 2000a; Nguyen *et al.*, 2002). Activation of the endothelium results in an upregulation of ICAM-1 and VCAM-1. Consistently, other adhesion receptors for transmigration of DCs are involved, like β2 and most probably the β1 integrins (chapter 5 and D'Amico *et al.*, 1998). A recent study showed defective migration of monocyte-derived DCs in LAD-1 immunodeficiency (Leukocyte Adhesion Deficiency) (Fiorini *et al.*, 2002). Patients suffering from LAD have a lost expression of β2 molecules (Anderson & Springer, 1987). Chemokine-induced migration as well as spontaneous migration through activated endothelium are impaired in LAD-1 patients, despite the fact that their DCs do express DC-SIGN (Fiorini *et al.*, 2002). This again indicates that β2 integrins are also important players in transmigration of DCs through activated endothelium. Since DC-SIGN binding to different types of endothelium seems to depend on the glycosylation of ICAM-2 on the distinct origins of endothelium (unpublished results van Kooyk & Geijtenbeek, 2002), rolling, adhesion and transmigration experiments of DCs through endothelium derived from different tissues will provide more insight into DC-SIGN-mediated migration to distinct sites in the body.

Though we found a major involvement of DC-SIGN in DC adhesion to endothelium, other studies showed that αLβ2 is equally important (D'Amico *et al.*, 1998; Nguyen *et al.*, 2002). Since αLβ2 requires activation to interact with its ligands (van Kooyk *et al.*, 1989; Dustin & Springer, 1989), these DC migration studies suggest that αLβ2 is in an active conformation on DCs. Staining of our DCs with an antibody which recognizes the activation epitope of αLβ2 showed that αLβ2 is not in an active state on DCs (unpublished results). Since activation of αLβ2 can be achieved by signaling via other adhesion molecules (Chan *et al.*, 2000; May *et al.*, 2000; Rose *et al.*, 2001), it would be interesting to study whether DC-SIGN could play a role in this activation process.

DC-SIGN is expressed on monocyte-derived and $CD34^+$-derived DCs upon culture with IL-4 and GM-CSF (Geijtenbeek *et al.*, 2000a). *In vivo*, DC-SIGN is present on DCs in many different tissues. Intriguingly, two DC-SIGN-positive cell populations are found in blood, a $CD14^-$ as well as a $CD14^+$ population (chapter 5). This latter population is of myeloid origin, since these cells express CD14, CD16, and CD33, in addition to CD1c, CD11b, CD11c, CD86, and high levels of MHC class I and II molecules (Engering *et al.*, 2002a). In addition, a subpopulation of BDCA-2^+ plasmacytoid DC (PDC) precursors expresses DC-SIGN (Soilleux *et al.*, 2002). In mouse, five homologues of DC-SIGN are cloned, one of which is highly expressed on DCs (Park *et al.*, 2001). The occurrence of DC-SIGN-positive cells in the periphery implies a role for DC-SIGN/ICAM-2 interactions in migration of these cells from blood into peripheral tissues and subsequently via lymph into lymph nodes. This hypothesis remains to be validated by *in vivo* experiments. The very low percentage of DC-SIGN-positive cells in blood, i.e. 0.04% of total PBMC (Chapter 5), makes these experiments difficult to perform.

DC-SIGN not only functions as an adhesion receptor, but also as an antigen receptor. DC-SIGN binds both viral and nonviral pathogens, including HIV, SIV, Mycobacterium tuberculosis, and Candida Albicans (Geijtenbeek *et al.*, 2000b; Pohlmann *et al.*, 2001; Appelmelk *et al.*, 2003; Cambi *et al.*, 2003; Tailleux *et al.*, 2003). Upon antigen binding, the DC-SIGN-antigen complex is internalized and targeted to endosomal compartments (Engering *et al.*, 2002b; Cambi *et al.*, 2003; Tailleux *et al.*, 2003). The dual function of DC-SIGN as an adhesion and antigen receptor raises the question whether pathogen uptake by DCs via DC-SIGN interferes with its adhesion and migration capacity. Previous studies done by Engering and colleagues indicate that antigen internalization mimicked by binding of DC-SIGN antibodies to DC-SIGN does not hinder ICAM-3 binding or T cell proliferation (Engering *et al.*, 2002b). Upon internalization of these anti-DC-SIGN/DC-SIGN complexes the surface expression of DC-SIGN is restored. However, HIV-1 infection results in the expression of HIV Nef protein, which affects the intracellular trafficking of DC-SIGN and enhances its cell surface expression (Sol-Foulon *et al.*, 2002). This enhanced expression results in increased adhesion to ICAM-3 expressed on T cells or ICAM-3-coated beads. Since the affinity of DC-SIGN for ICAM-2 is higher than for ICAM-3 (chapter 5), it is very likely that Nef also enhances DC-SIGN/ICAM-2 interactions. This increased adhesion might affect migration of DCs to lymphoid organs resulting in an impaired immune response. The findings of Engering and Sol-Foulon and colleagues suggest that each pathogen might activate different signaling pathways upon DC-SIGN binding. More experiments will be required to unravel the effects of DC-SIGN-mediated antigen uptake on DC adhesion and transendothelial migration.

After pathogen uptake DCs mature and migrate to lymph nodes. *In vitro*, maturation of immature DCs with TNFα slightly reduced DC-SIGN expression. A functional role for this

downmodulation of DC-SIGN has not been described. Future experiments will have to reveal whether the reduced expression of DC-SIGN on mature DCs affects DC-SIGN-mediated migration.
A recent study unraveling the carbohydrate specificity of DC-SIGN showed that Le^x-neoglycoconjugates, such as Le^x, Le^a, Le^y, Le^b, and sulfo-Le^a, all bind with high affinity to DC-SIGN (Appelmelk *et al.*, 2003). Le^a is present on mucin-expressing tumors (reviewed in Appelmelk *et al.*, 2003), whereas Le^y is overexpressed on many carcinomas, including ovary, pancreas, prostate, breast, colon, and non-small cell lung cancers (Hellstrom *et al.*, 1990). This indicates that DC-SIGN may mediate adhesion between DCs and tumor cells. The binding of DC-SIGN to tumor peptides might be of great relevance in anti-tumor therapy, in particular for non-immunogenic tumors. DC-SIGN could be relevant for tumor antigen capture and internalization, which is followed by processing and subsequent peptide presentation in MHC molecules. In this way, tumor antigen uptake via DC-SIGN might induce antitumor responses. DC-SIGN positive cells are found in the peritumoral area of melanomas (Vermi *et al.*, 2003). This suggests a role for DC-SIGN in tumor antigen uptake. Hence, it would be very interesting to test the direct interaction of tumor cells or tumor antigens with DCs via DC-SIGN. It is important to realize that solid malignancies overexpress TGF-β1. TGF-β1 suppresses the immune system by restraining *in vitro* activation and upregulation of costimulatory molecules, resulting in an arrest in DC maturation and reduction in antigen presentation (Strobl & Knapp, 1999). Whereas human DCs derived from $CD34^+$ progenitors are blocked in their maturation upon TGF-β1 exposure, epithelial Langerhans Cells are positively regulated by TGF-β1. For example, BL6 murine melanoma metastasized animals have high plasma levels of TGF-β1, and TGF-β1 and TGF-β2 plasma levels are significantly higher in colon cancer patients compared with unaffected individuals (La Porta & Comolli, 2000; Bellone *et al.*, 2001). TGFβ is able to reduce DC-SIGN expression (Relloso *et al.*, 2002). This suggests that TGFβ might affect DC adhesion to tumors and tumor endothelium and subsequent migration into malignant tissue via downregulation of DC-SIGN expression.

CD83

The maturation process of DCs is characterized by an upregulation of costimulatory molecules, MHC molecules, and of the maturation marker CD83. The selective expression of CD83 on mature DCs suggests a functional role of CD83 in immune responses. Indeed, inhibition of CD83 protein synthesis results in reduced T cell stimulation by DCs (Kruse *et al.*, 2000a). Circumstantial evidence for a biological role of CD83 came from studies in which DCs were infected with viruses (Kruse *et al.*, 2000b; Jenne *et al.*, 2000). For instance, HSV-1 infection of DCs results in degradation of CD83 resulting in a reduced capacity to initiate T cell proliferation in an allogeneic mixed leukocyte reaction setting (Kruse *et al.*, 2000b). The importance of CD83 in immune responses is also underscored in CD83-/- mice, which showed a block in $CD4^+$ single positive thymocyte generation (Fujimoto *et al.*, 2002).
Normal human sera contain low levels (121 ± 3.6 pg/ml) of a circulating soluble form of CD83, which is released from activated DCs and B lymphocytes (Hock *et al.*, 2001). We demonstrated that both immature and mature DCs bind to hCD83-Fc fusion protein (chapter 6). Immature DCs lack CD83 suggesting that the interaction of CD83 with DCs is heterophilic. Scholler and colleagues showed that CD83-Ig binds to monocytes and a subset

of activated $CD8^+$ T cells (Scholler *et al.*, 2002). This again indicates a heterophilic interaction of CD83. Because monocytes, a subset of activated $CD8^+$ T cells, immature and mature DCs all bind CD83 a CD83 receptor shared by those cells might be involved in this interaction. Another possibility is that those cells use different receptors. Future experiments using blocking antibodies or biochemical reagents have to reveal which receptor or kind of receptor is involved.

Addition of the soluble recombinant extracellular domain of CD83 (hCD83ext) to immature DCs resulted in a block in DC maturation, as indicated by a reduction in CD80 and CD83 surface expression (chapter 6). The expression of MHC class I and II as well as CD86 were not affected by hCD83ext. Since DC maturation is a prerequisite for DC migration into T cell areas of lymph nodes (chapter 3), it would be interesting to investigate whether DCs treated with soluble CD83 also lack expression of the chemokine receptor CCR7 and if the random and chemotactic migratory behavior of these DCs is impaired. The migration of partially matured DCs into lymph nodes might result in a non-effective, suppressive immune response.

Next to the block on DC maturation, hCD83ext inhibits mature DC-induced allogeneic and peptide-specific T cell proliferation in a concentration-dependent manner (chapter 6). Scholler and colleagues showed that soluble CD83-Ig fusion protein is costimulatory when coimmobilized with anti-CD3 (Scholler *et al.*, 2002). As described above, human sera contain low amounts of soluble CD83. We used 10,000 fold higher concentrations than those observed in human sera to demonstrate the block in DC maturation and inhibition of T cell stimulation. It would therefore be very interesting to investigate soluble CD83 concentrations in serum in pathological situations to validate the (patho)physiological relevance of this circulating factor. Intraperitoneal injection of soluble CD83-Ig in tumor-bearing mice results in larger tumors than in the control group (Scholler *et al.*, 2002). This suggests that if high concentrations of soluble CD83 are present, this may lead to immunosuppression. The measurement of soluble CD83 concentrations in the environment of activated DCs may also provide clues to the physiological function of CD83. High local CD83 concentrations might control the immune response by inducing an arrest in DC maturation. Future studies unraveling the receptor for CD83 on DCs might clarify the signaling mechanisms underlying this inhibitory effect. In addition, these investigations will reveal whether distinct or the same CD83/receptor interactions are used for DC adhesion, inhibition of DC maturation and T cell proliferation.

Arrode and colleagues described an indirect effect of virus on CD83 expression. Cross-presentation of human cytomegalovirus (HCMV) late-stage infected fibroblasts results in inhibition of DC maturation (Arrode *et al.*, 2002). Soluble factors secreted by HMCV-infected fibroblasts modulate CD83 expression on DCs, resulting in a reduced capacity to induce an antiviral $CD8^+$-T cell response. Transforming Growth Factor-β1 (TGF-β1) is one of the modulators involved, though the authors cannot exclude IL-10 as an interfering cytokine in DC maturation.

As described above cancer patients have higher plasma levels of TGFβ than unaffected individuals. This aspect together with the downmodulating effect of TGFβ on CD83 expression and subsequent inhibition of DC maturation might reduce anti-tumor responses by suppressing the costimulatory effect of CD83. Mouse melanoma cells transfected with CD83 induce an anti-tumor response against wild-type melanoma cells, whose immunogenicity is very low (Scholler *et al.*, 2002). Scholler and colleagues tentatively

conclude that interaction between CD83 and its ligand(s) may be involved in regulation of immune response to tumors by activation of $CD8^+$ T cells. The transfection of CD83 into tumor cells might be an interesting way to increase the anti-tumor response. A more profound knowledge of the binding partners and the costimulatory function of CD83 could lead to new insights in tumor immunology and new therapeutic strategies.

OTHER RECEPTORS

DC migration is directed by chemokines and their receptors. Upon maturation, DCs switch their chemokine receptor repertoire and express CCR7. CCR7 is required for the mobilization of mature DCs into lymphatic vessels and their subsequent entrance in secondary lymphoid organs. In chapter 3, we demonstrated that mature DCs, which do express CCR7, indeed migrate into T cell areas of lymph nodes. Immature DCs labeled with ^{111}In, which do not express CCR7 were also able to reach the lymph nodes, but did not enter the T cell areas. Unfortunately, we were not able to stain the ^{111}In-labeled DCs in situ with CD83 or CCR7 antibodies, since the ^{111}In-staining was too prominent. The fact that immature DCs did reach the lymph nodes could not be contributed to an *in vivo* maturation process, since these DCs were not able to induce a *"de novo"* immune response *in vivo*. The localization of injected immature DCs at marginal sinuses in lymph nodes after intranodal injection suggests that those DCs, which lack CCR7, do emigrate from the injection depot. This might be due to the disruption of the microenvironment after injection or to passive trafficking via the flow of lymph vessels.
Other receptors, which are indirectly involved in DC migration, are EP2 and EP4. These receptors bind PGE_2, which is a cofactor in DC differentiation, induces the expression of CCR7 on DCs, and is an important modulator of their migratory function (Jonuleit *et al.*, 1997; Kalinski *et al.*, 1998; Luft *et al.*, 2002; Scandella *et al.*, 2002). Interestingly, we and others observed a reduced random migration of DCs when matured in the presence of MCM, TNFα, but in the absence of PGE_2 (unpublished data; Jonuleit *et al.*, 1997). This must be an effect of maturation because PGE_2 by itself does not induce random migration (K. Broers unpublished data). Serum contains many chemokines and lipids, like ATP, sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA), which can induce cell migration (Idzko *et al.*, 2002; Panther *et al.*, 2002; Idzko *et al.*, 2002). Since we performed our experiments in the presence of serum, it is tempting to speculate that these components induce DC migration in our system. Preliminary migration experiments using overnight serum-starved mature DCs in medium containing albumin, showed migration, indicating migration as an intrinsic property of the cells. However, other preliminary experiments showed that culturing of DCs in the absence of serum abrogates DC migration. These data suggest that presence of serum factors during DC culture and a maturation cocktail including PGE2 are prerequisite for random DC migration.

LINK OF DC MIGRATION TO ANTI-TUMOR THERAPY AND FUTURE PERSPECTIVES

Nowadays DCs are used as cell-based vaccines in immunotherapy against cancer. Irrespective of the place of injection (subcutaneously, intradermally, intraveneously, or

intranodally), these DCs must come in close proximity of T cells in the lymph nodes. Mature DCs are preferred over immature DCs since they have higher migratory capacities and do reach the T cell areas of the lymph nodes, where they can efficiently stimulate naïve T cells (Hollender *et al.*, 2002; Gunzer *et al.*, 2003; de Vries *et al.*, 2003). In contrast, immature DCs can silence T cells either by deletion or by inducing regulatory T cells, which exert suppressive immune functions (reviewed in Steinman *et al.*, 2003). The presence of regulatory T cells downmodulates the anti-tumor response.

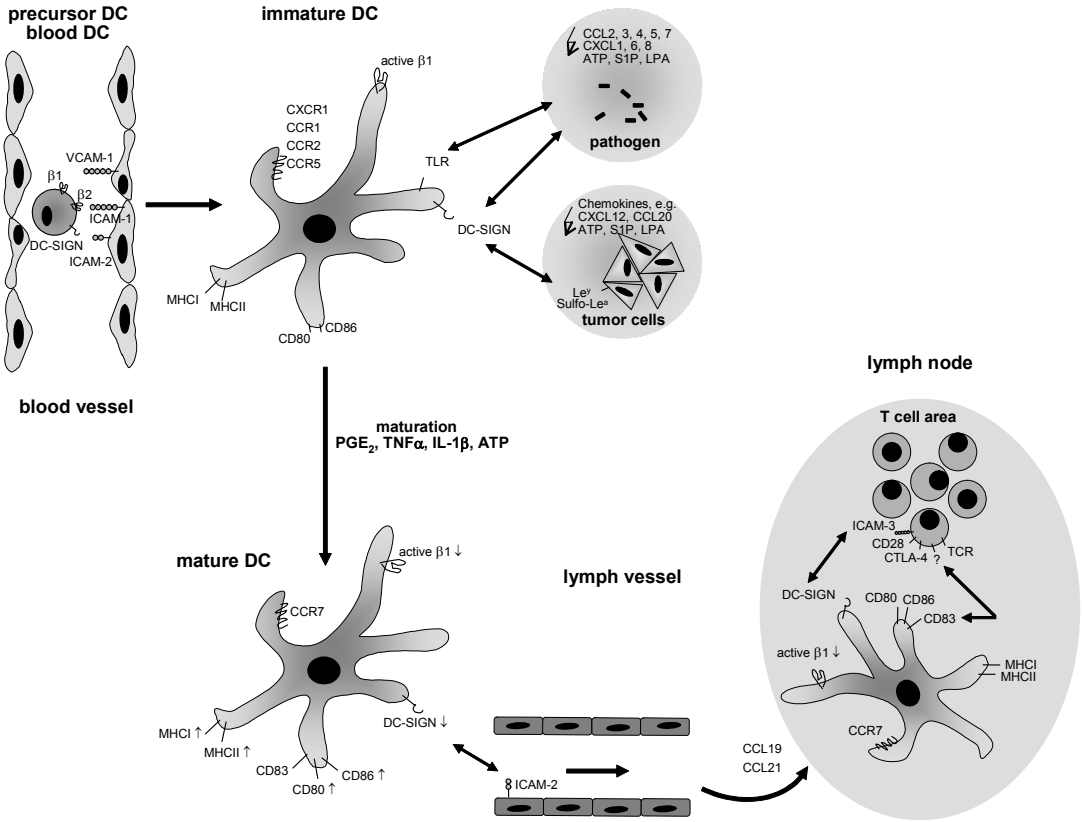

Figure 3. DC migration model. The migration of DC precursors and blood DC into peripheral tissues is presumably mediated via DC-SIGN-ICAM-2 interactions. When the tissue is inflamed other receptors, like β1 and β2 integrins are also involved. Subsequently, these DCs reside in an immature state expressing low MHC molecules, low costimulatory molecules, DC-SIGN and active β1 integrins, and patrol for antigen. Upon sensing chemokine gradients, but also LPA, S1P, and ATP, they migrate to the center of these gradients and pick up pathogens either via DC-SIGN, toll-like receptors or via endocytotic pathways. In case of tumors, they might migrate into malignant tissue via DC-SIGN-tumor endothelium interactions and/or adhere to tumor cells via DC-SIGN-Le^y or sulfo-Le^a interactions. Danger signals, such as PGE_2, TNFα, IL-1β, but also ATP induce maturation of immature DCs; MHC molecules and costimulatory molecules are upregulated, CCR7 and CD83 are induced, the cytoskeleton will rearrange and active β1 integrins are downmodulated, as is DC-SIGN. Mature DCs migrate via lymph vessels (DC-SIGN-ICAM-2 interactions) into T cell areas of lymph nodes via CCR7. They interact with naïve T cells via DC-SIGN-ICAM-3 interactions and activate these T cells via costimulatory molecules and CD83.

Although injected mature DCs do express CCR7, a major proportion remains in the injection depot. Enhanced migration of DCs out of this depot by the induction of matrix metalloproteinases might induce more effective T cell priming (Ratzinger *et al.*, 2002). In

addition, the application of inflammatory cytokines at the place of injection might induce the migration of those DCs. Interestingly, more data becomes available that the injection site is also very important for T cell priming and homing. It becomes clear that T cells stimulated in a lymph node gain local homing receptors to exert their effect in the environment of that particular lymph node (Dudda, 2003; Mora *et al.*, 2003). This suggests that local administration of DCs results in local homing receptors on T cells. This finding is of importance for DC-based anti-tumor therapy, in which activated T cells should specifically migrate to and attack tumor cells.

In conclusion, many factors determine whether DC-based vaccines are able to migrate and are effective in anti-tumor therapy. We demonstrated that DC migration is most prominent in mature DCs. In addition, we showed that the distinct adhesive behavior between immature and mature DCs is regulated by the activation state of the β1 integrin chain. In this respect immature DCs have higher expression levels of active β1 and likewise adhere better to fibronectin than mature DCs. Future studies should reveal which cytoskeletal component or transmembrane protein causes this difference in activation state of β1 integrin. We also showed that DC adhesion and migration through endothelium are mediated by DC-SIGN. However, *in vivo* data are still lacking. Another molecule studied in this thesis is CD83. By using CD83-Fc, we demonstrated that DCs adhere to this chimera and that binding results in a block in DC maturation and T cell proliferation. Although soluble CD83 was measured in serum, its *in vivo* role remains to be determined. A schematic overview of the role of β1-integrins, CCR7, DC-SIGN, and CD83 in DC biology is depicted in Fig. 3. I want to stress that this figure only focuses on the molecules presented in this thesis and that many more molecules and proteins are involved. *In vivo*, DC adhesion and migration depend on numerous environmental factors that until now cannot be replicated by *in vitro* studies. The introduction of two-photon imaging allowed the discrimination of individual cells in their natural environment, e.g. in the lymph nodes. This technique is very promising to study DC behavior in physiological cellular compartments and might expand our knowledge of individual migration capacities of cells.

Summary
Nederlandse samenvatting

SUMMARY

The immune system is a very complex system, which controls the body for invading microorganisms, like bacteria and viruses (pathogens). This defense system comprises many different white blood cells, also called leukocytes that orchestrate to fight against pathogens. Dendritic cells (DCs) and T cells are important players in the defense system. DC precursors are produced in the bone marrow, circulate in the blood and pass the blood vessel wall (endothelium) to enter tissues (transendothelial migration). Once in the tissues, these precursors develop into immature DCs, which patrol for invading pathogens. Upon pathogen encounter, DCs take up the pathogen, degrade the proteins into small pieces, called peptides and present these peptides in specialized molecules (MHC molecules) on their surface. This process is accompanied by a change in DC phenotype; they transit from an immature into a mature state. Upon maturation DCs migrate from the infection site via lymph vessels into the lymph nodes. Here they interact and present the MHC-peptide complexes to naïve T cells. Recognition of the complex by T cells with the appropriate receptor results in T cell activation. Activated T cells proliferate and subsequently leave the lymph nodes and migrate via the blood into the infected tissue, where they can directly kill the infected cells.

During their migration DCs and T cells continuously interact with endothelium and tissues. These interactions are mediated by cell adhesion molecules. Many cell adhesion molecules are present on the surface of leukocytes. They can be divided in several groups, including lectins and integrins. Integrins mediate a broad range of interactions between cells (β1 and β2 integrins) or between cells and tissue structures (β1 integrins). Cell adhesion via integrins is tightly regulated. The cell can either change the affinity of the integrin (binding force of the integrin) or the avidity of the integrin (lateral mobility of integrins induced by changes in the skeleton of the cell (cytoskeleton)) to bind its specific counterpart molecules (ligands). In this thesis the role of integrins in DC and T cell adhesion and migration is investigated. In addition, the function of an adhesion molecule of the lectin group, specifically expressed on DCs, called DC-SIGN in adhesion and migration is studied. Finally, the cell surface molecule CD83 and its role in DC adhesion and the immune response is studied.

Chapter 1 provides an overview of the adhesion molecules that are involved in adhesion and migration of DCs and T cells through endothelium and within tissues. This chapter describes the state of the art on the role of integrins and lectins (like DC-SIGN) in these processes and the scope of this thesis.

Many techniques are available to study leukocyte migration in the lab. Some of them only provide information on the total cell population, whereas more sophisticated migration assays coupled to time-lapse video systems monitor the migratory behavior of individual cells. The cell recognition in these latter systems is based on contrast differences between cells and the contact surface or on *a priori* cell shape (morphology) criteria. As migrating cells continuously change their shape during their movement, the algorithm should be robust to the changing morphology. In **chapter 2**, an Automated Cell Track System (ACTS) to quantify and track migration of multiple cells is described, which fulfils the specifications of such an algorithm. Automatic tracking of these cells highly correlated with manual analysis. This novel system also allows simultaneous monitoring of multiple experimental conditions. The migration of T cells (HSB-2 T cells) on the InterCellular Adhesion Molecule-1 (ICAM-1) was used to validate the system. ICAM-1 is a cell surface molecule expressed on various cell types, including endothelium and functions as a ligand for integrins. HSB-2 T cells bind

ICAM-1 via the β2 integrin αLβ2. We show that activation of αLβ2 resulted in an enhanced binding and a decreased migration of HSB-2 T cells on ICAM-1. In addition, both the adhesion and migration of HSB-2 T cells depended on the ICAM-1 concentration.

DCs, as professional antigen presenting cells are nowadays explored for their capacity to present tumor antigens to the immune system and raise anti-tumor responses in patients. They are cultured in the laboratory from their precursors circulating in the blood, loaded with tumor peptides or proteins, and subsequently re-injected into the patient. To elicit a proper immune reaction against tumors, DCs have to migrate from the injection site to draining lymph nodes to interact and stimulate naïve T cells. In **chapter 3**, the adhesive and migratory behaviors of immature and mature DCs are compared. Immature DCs strongly adhered to the tissue protein fibronectin via the β1 integrin α5β1, whereas mature DCs were highly motile as measured by the ACTS. Interestingly, this difference in DC migration was also observed in our *in vivo* studies in melanoma patients, in which only mature DCs reached the T cell areas of lymph nodes. This highly migratory capacity together with the T cell-interacting properties favors mature DCs over immature DCs in immunotherapy against cancer.

The distinct adhesive characteristics of immature and mature DCs were further studied in **chapter 4**. As described DC adhesion to fibronectin is mediated by α5β1. Although immature and mature DCs expressed comparable levels of the integrin chain α5, mature DCs adhered considerably less to fibronectin than immature DCs. The reduced adhesion of mature DCs could be restored by activation of α5β1. Thus during DC maturation α5β1 becomes inactive and/or differentially distributed at the cell surface. The lower expression of the activation epitope 12G10 on mature DCs (presence of 12G10 indicates an active conformation of β1 integrins) confirmed this finding. Incubation of immature, but not mature DCs on fibronectin resulted in the formation of special adhesion structures (podosomes). These structures contained active β1 integrins and regulate the adhesion of immature DCs to fibronectin.

The extravasation of leukocytes from the blood into tissues is a multi-step process. First, leukocytes are slowed down and roll along the endothelium. Second, their integrins will be activated resulting in leukocyte arrest and adhesion to the endothelium. The adhesion is followed by transmigration, in which leukocytes pass the endothelium and enter the underlying tissue. In **chapter 5**, the role of DC-SIGN in DC adhesion and migration through endothelium is explored. DC-SIGN is specifically expressed on DCs and binds next to several pathogens like HIV, Candida Albicans, and Mycobacteria tuberculosis to ICAM-3. ICAM-3 has a high homology with ICAM-2, which is constitutively expressed on (lymph vessel) endothelium. We showed that DC-SIGN binds ICAM-2 and facilitates DC-endothelium interactions. To study the role of DC-SIGN in rolling on ICAM-2, we artificially induced expression of DC-SIGN in K562 cells (K-DC-SIGN), which normally do not have DC-SIGN on their surface. These K-DC-SIGN cells rolled on ICAM-2, indicating that DC-SIGN mediates rolling-interactions. In addition, we show that DC-SIGN regulated DC adhesion to endothelium and was involved in their transendothelial migration. These findings imply a role for DC-SIGN in DC extravasation.

Maturation of DCs is characterized by an upregulation of costimulatory and MHC molecules, molecules that form the complex structure for specific interaction with the T cells. In addition, mature DCs express the maturation marker CD83. In **chapter 6**, we assessed the

functional role of CD83. Immature as well as mature DCs, which do express CD83, bound to CD83. Since immature DCs lack CD83, the interaction should be heterophilic (mediated by a distinct adhesion receptor). Binding of CD83 by DCs resulted in a maturation block as indicated by a reduced expression of the costimulatory molecule CD80 and the maturation marker CD83. In addition, a reduction in T cell activation was found after treatment of DCs with CD83. Interestingly, CD83 had no effect on the phenotype of the T cells.
The activation state of integrins determines cell adhesion and migration, as illustrated in this thesis. It has become apparent that several cytoskeletal proteins and membrane proteins regulate integrin activation. In **chapter 7** the role of the cytoskeleton, but also of membrane proteins on integrin activation is discussed. Future investigations will have to shed light whether these proteins are differentially regulated in immature versus mature DCs and cause their distinct adhesive capacities. In addition, the function of DC-SIGN as an adhesion and antigen receptor, and the functional role of CD83 are discussed in this chapter.

NEDERLANDSE SAMENVATTING

Het immuunsysteem is een erg complex systeem dat op zoek gaat naar allerlei micro-organismen die het lichaam binnengedrongen zijn, zoals bacteriën en virussen (pathogenen). Dit verdedigingssysteem bestaat uit veel verschillende witte bloedcellen, ook wel leukocyten genoemd die samenwerken om tegen pathogenen te vechten. Dendritische Cellen (DCs) en T cellen zijn belangrijke spelers van het afweersysteem. Voorlopers van DCs worden geproduceerd in het beenmerg. Ze circuleren in het bloed en moeten de bloedvatwand (endotheel) passeren alvorens ze het onderliggende weefsel binnen kunnen treden (transendotheliale migratie). Eenmaal in het weefsel, ontwikkelen voorloper DCs zich tot onrijpe DCs, die op zoek gaan naar binnengedrongen pathogenen. Wanneer ze een pathogeen gevonden hebben, nemen ze deze op, verwerken het tot kleine deeltjes (peptiden genoemd) en presenteren deze peptide in gespecialiseerde moleculen (MHC moleculen) op hun celoppervlak. Dit proces gaat gepaard met een verandering in DC fenotype; ze gaan van een onrijpe naar een rijpe vorm. Vervolgens migreren de rijpe DCs via lymfevaten naar de lymfeklieren. Hier gaan ze een interactie aan met naïve T cellen en presenteren ze de MHC-peptide complexen. Herkenning van het complex door de T cel resulteert in T cel activatie. Geactiveerde T cellen vermenigvuldigen zich, verlaten vervolgens de lymfeklieren en migreren via het bloed naar het geïnfecteerde weefsel, waar ze de geïnfecteerde cellen direct kunnen doden.
Tijdens hun migratie gaan DCs en T cellen interacties aan met endotheel en weefsels. Deze interacties worden verzorgd door adhesiemoleculen. Het oppervlak van leukocyten bevat veel adhesiemoleculen. Ze kunnen verdeeld worden in verschillende groepen, waaronder lectines en integrines. Integrines reguleren verschillende interacties tussen cellen (β1 en β2 integrines) of tussen cellen en weefselstructuren (β1 integrines). Celadhesie door integrines is strikt gecontroleerd. De cel kan de affiniteit van het integrine veranderen (bindingskracht van het integrine) of de aviditeit van het integrine (verschuiving van integrines over de celmembraan door veranderingen in het skelet van de cel (cytoskelet)) om zijn tegenstructuur (liganden) te binden. In dit proefschrift is de rol van integrines in DC en T cel adhesie (binding) en migratie bestudeerd. Tevens is de functie van een adhesiemolecuul van de lectinegroep, genaamd DC-SIGN, bestudeerd in adhesie en migratie. Tot slot is de rol van het oppervlakte-eiwit CD83 in DC adhesie en de immuunrespons onderzocht.
Hoofdstuk 1 geeft een overzicht van de adhesiemoleculen die betrokken zijn bij de adhesie en migratie van DCs en T cellen door endotheel en in weefsels. Dit hoofdstuk beschrijft de rol van integrines en lectines (zoals DC-SIGN) in deze processen en het doel van dit proefschrift.
Er zijn veel technieken waarmee leukocytmigratie in het lab bestudeerd kan worden. Sommige van deze technieken geven slechts informatie over de totale celpopulatie, terwijl meer geavanceerde migratiesystemen, die gekoppeld zijn aan time-lapse video's, het migratiegedrag van individuele cellen vastleggen. In deze laatst genoemde systemen wordt de cel herkend op basis van contrastverschillen tussen de cel en zijn contactoppervlak of op *a priori* celvorm (morfologie) criteria. Het algoritme wat gebruikt wordt moet echter robuust zijn voor deze morfologieveranderingen aangezien bewegende cellen continu van vorm veranderen. In **hoofdstuk 2** wordt een automatisch celtrack systeem (ACTS) beschreven dat voldoet aan de specificaties voor dit algoritme en waarmee meerdere cellen gekwantificeerd en gevolgd kunnen worden tijdens hun migratie. Het automatisch traceren

van de cellen correleerde erg goed met manuele analyses. Daarnaast kunnen met dit nieuwe systeem verschillende experimentele condities simultaan gevolgd worden. De migratie van T cellen (HSB-2 T cellen) op het InterCellulair AdhesieMolecuul-1 (ICAM-1) is gebruikt om het systeem te valideren. ICAM-1 is een celoppervlaktemolecuul dat in verschillende cellen tot expressie wordt gebracht, inclusief endotheel, en is een ligand voor integrines. HSB-2 T cellen binden aan ICAM-1 via het β2 integrine αLβ2. Wij laten zien dat activatie van αLβ2 resulteerde in een verhoogde adhesie en een verminderde migratie van HSB-2 T cellen op ICAM-1. Tevens was de adhesie en migratie van HSB-2 T cellen afhankelijk van de ICAM-1 concentratie.

DCs worden als professioneel antigen-presenterende cellen gebruikt vanwege hun capaciteit om tumorantigenen te presenteren aan het immuunsysteem en wekken antitumorresponsen op in patiënten. Ze worden in het laboratorium gekweekt van de DC-precursors die in het bloed circuleren, beladen met tumorpeptiden of eiwitten, en vervolgens weer geïnjecteerd in de patiënt. Om een goede immuunreactie tegen tumoren te bewerkstelligen moeten de geïnjecteerde DCs van de injectieplaats naar de lymfeklieren migreren om naïve T cellen te ontmoeten en te stimuleren. In **hoofdstuk 3** worden het adhesie- en migratiegedrag van onrijpe en rijpe DCs vergeleken. Onrijpe DCs binden aan het weefseleiwit fibronectine via het β1 integrine α5β1, terwijl rijpe DCs juist migreren op fibronectine (gemeten met het ACTS). Dit verschil in DC migratie werd ook bevestigd door *in vivo* studies in melanoompatiënten, waarin alleen rijpe DCs de T celgebieden van de lymfeklieren bereikten. Hun goede migratiegedrag en hun capaciteit om interacties aan te gaan met T cellen maken rijpe DCs favoriet in immunotherapie tegen kanker.

Het verschil in adhesiegedrag tussen onrijpe en rijpe DCs is verder uitgediept in **hoofdstuk 4**. Zoals zojuist beschreven wordt de adhesie van DCs aan fibronectine gereguleerd door α5β1. Ondanks dat onrijpe en rijpe DCs vergelijkbare hoeveelheden β1 tot expressie brengen hechten rijpe DCs aanzienlijk minder aan fibronectine dan onrijpe DCs. Deze lage adhesie kon hersteld worden door β1 te activeren. Dit impliceert dat β1 minder actief is en/of anders verdeeld is over het celoppervlak van rijpe DCs vergeleken met onrijpe DCs. De lage expressie van de activatie-epitoop 12G10 op rijpe DCs (aanwezigheid van 12G10 indiceert een actieve vorm van β1 integrines) bevestigde dit. Incubatie van onrijpe, maar niet van rijpe DCs op fibronectine resulteerde in de vorming van speciale adhesiestructuren (podosomen). Deze structuren bevatten actieve β1 integrines en reguleren de adhesie van onrijpe DCs aan fibronectine.

De migratie van leukocyten vanuit het bloed naar het onderliggende weefsel is een proces dat uit verschillende stappen bestaat. Eerst worden de leukocyten afgeremd en rollen ze over het endotheel. Vervolgens worden hun integrines geactiveerd wat resulteert in een stopsignaal en adhesie aan het endotheel. De adhesie wordt gevolgd door de transmigratiestap, waarbij leukocyten het endotheel passeren en het onderliggende weefsel binnentreden. In **hoofdstuk 5** wordt de rol van DC-SIGN in DC adhesie en migratie door endotheel beschreven. DC-SIGN komt specifiek tot expressie op DCs en bindt naast allerlei pathogenen, zoals HIV, Candida Albicans en Mycobacterium Tuberculosis, aan ICAM-3. ICAM-3 heeft grote overeenkomsten met ICAM-2 dat zich op endotheel in bloed- en lymfevaten bevindt. Wij laten zien dat DC-SIGN aan ICAM-2 bindt en zo DC-endotheelinteracties kan reguleren. Om de functie van DC-SIGN in het rollen over endotheel te bestuderen, hebben we artificieel DC-SIGN tot expressie gebracht in K562 cellen (K-DC-SIGN), die normaal geen DC-SIGN op hun oppervlak hebben. Deze K-DC-SIGN cellen rollen

op ICAM-2 wat betekent dat DC-SIGN rolinteracties kan reguleren. Tevens reguleerde DC-SIGN de adhesie van DCs aan endotheel en was het betrokken bij de transendotheliale migratie van DCs. Deze bevindingen impliceren een rol voor DC-SIGN in de uittreding van DCs uit bloed- en lymfevaten.
De rijping van DCs wordt gekarakteriseerd door een verhoogde expressie van costimulatoire en MHC moleculen, moleculen die een complexe structuur vormen voor specifieke interacties met T cellen. Daarnaast brengen rijpe DCs ook het eiwit CD83 tot expressie. In **hoofdstuk 6**, hebben we de functionele rol van CD83 bestudeerd. Zowel onrijpe DCs, die geen CD83 tot expressie brengen, als rijpe DCs bonden aan CD83. Omdat onrijpe DCs geen CD83 op hun oppervlak hebben, moet de interactie wel heterofiel zijn (verzorgd door een andere adhesiereceptor). De binding van CD83 aan DCs resulteerde in een blokkade in het rijpingsproces. Dit werd gekenmerkt door een gereduceerde expressie van het costimulatoire molecuul CD80 en de rijpingsmarker CD83. Eveneens werd een verminderde T cel stimulatie waargenomen na behandeling van de DCs met CD83. CD83Fc had echter geen effect op het fenotype van T cellen.
Zoals geïllustreerd wordt in dit proefschrift bepaalt de mate van integrine-activatie celadhesie- en migratie. Het is bekend dat verschillende cytoskeletaire eiwitten en membraaneiwitten integrine activatie reguleren. In **hoofdstuk 7** wordt de rol van cytoskeletaire eiwitten, maar ook membraaneiwitten op integrine activatie bediscussieerd. In de toekomst zullen studies moeten bepalen of deze eiwitten verschillend gereguleerd zijn in onrijpe versus rijpe DCs en of ze het verschil in adhesie veroorzaken. Daarnaast worden in dit hoofdstuk de functie van DC-SIGN als een adhesie- en antigenreceptor en de functionele rol van CD83 bediscussieerd.

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ACTS	automated cell track system
APC	antigen-presenting cell
Arp	actin-related protein
ATP	adenosine triphosphate
BDCA	blood dendritic cell antigen
CD	cluster of differentiation
CLA	cutaneous lymphocyte-associated antigen
CLSM	confocal laser scanning microscopy
CTL	cytotoxic T lymphocyte
CYTIP	cytohesin-1 interacting protein
DC	dendritic cell
DC-SIGN	dendritic cell specific ICAM-grabbing non-integrin
DTH	delayed type hypersensitivity
ECM	extracellular matrix
ELC	EBI1-ligand chemokine
ELISPOT	enzyme linked immunospot assay
ESL-1	E-selectin ligand-1
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
GM-CSF	granulocyte/macrophage colony stimulating factor
gp	glycoprotein
GST	glutathion S transferase
HCMV	human cytomegalovirus
HEV	high endothelial venule
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HMEC	human microvascular endothelial cell
HSV	herpes simplex virus
ICAM	intercellular adhesion molecule
ICAP-1	integrin cytoplasmic domain-associated protein-1
ILK	integrin-linked kinase
IFN	interferon
Ig	immunoglobulin
^{111}In	111-Indium
IL	interleukin
JAM	junctional adhesion molecule
KLH	keyhole limpet hemocyanin
LAD	leukocyte adhesion deficiency
LARC	liver- and activation-regulated chemokine
LC	Langerhans cell
Le	Lewis neoglycoconjugate
LFA-1	leukocyte function-associated antigen-1
LPA	lysophosphatidic acid

LTC4	leukotriene C4
mAb	monoclonal antibody
Mac-1	macrophage receptor
MAdCAM-1	mucosal addressin cell adhesion molecule-1
MCM	monocyte conditioned medium
MDC	myeloid dendritic cell
MDR	multidrug resistance protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLR	mixed lymphocyte reaction
MMR	macrophage mannose receptor
MRP	multidrug resistance-associated protein
NK	natural killer cell
NMR	nuclear magnetic resonance
PBMC	peripheral blood mononuclear cell
PBL	peripheral blood lymphocyte
PCR	polymerase chain reaction
PDC	plasmacytoid dendritic cell
PE	(R-)phycoerythrin
PGE_2	prostaglandin E2
PKC	protein kinase C
PLC	phospholipase C
PSGL-1	P-selectin glycoprotein ligand-1
Rack1	receptors for activated C-kinase
RANTES	regulated upon activation, normal T expressed, and secreted
RNA	ribonucleic acid
S1P	sphingosine-1 phosphate
SDF-1	stromal cell-derived factor-1
SDS	sodium dodecyl sulphate
SIV	simian immunodeficiency virus
SLC	secondary lymphoid tissue chemokine
Tcm	central memory T cell
Tem	effector memory T cell
TGF	transforming growth factor
Th cell	T helper cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule-1
VLA	very late antigen
WASp	Wiskott-Aldrich syndrome protein

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CURRICULUM VITAE

Daniëlle Krooshoop werd geboren op 11 oktober 1973 te Goor. In 1992 behaalde zij haar VWO diploma aan het R.K. Lyceum de Grundel te Hengelo (o). In datzelfde jaar begon zij aan de studie biomedische gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen. Tijdens haar studie heeft zij als stagiaire bij de afdeling Cytogenetica van het UMC St. Radboud onder begeleiding van F. Stellink, dr. J. Tuerlings en Prof. dr. A. Geurts van Kessel onderzoek gedaan naar de detectie van microdeleties van 9p21 van MTS1 en MTS2 genen bij lymfomen. Daarna heeft zij stage gelopen op de afdeling immunologie van het Nederlands Kanker Instituut onder supervisie van dr. M.A. Oosterwegel en Prof. dr. A.M. Kruisbeek. Hier heeft ze met behulp van de phage-display techniek onderzoek uitgevoerd naar de identificatie van antigenen op stromale cellen die mogelijk betrokken zijn bij T cel ontwikkeling. Als extra stage is zij in 1997 voor 7 maanden naar Cambridge in Engeland geweest. Op de afdeling Protein and Nucleic Acid Chemistry van het Medical Research Council heeft zij onder begeleiding van dr. A.E. Corcoran en dr. A.R. Venkitaraman de rol van de transcriptiefactor pax5 tijdens de regulatie van de B cel ontwikkeling door de interleukine 7 receptor bestudeerd. Tijdens haar studie heeft ze in 1994 de aantekening proefdierdeskundige (ex. Art. 9 van de Wet op Dierproeven) behaald. Na het afstuderen begon zij in september 1997 als Assistent in Opleiding aan de afdeling tumorimmunologie van het UMC Nijmegen onder leiding van Prof. dr. C.G. Figdor aan het in dit proefschrift beschreven onderzoek.

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